001

The Cytoplasmic Domain of Bullous Pemphigoid Antigen 180 (BP180) Interacts with the Globular Head Domain of Bullous Pemphigoid Antigen 230 (BP230)

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Hemidesmosomes (HD) are junctional complexes that play a key role in promoting dermo–epidermal cohesion. Our understanding of the molecular organization of these multiprotein complexes remains incomplete. The bullous pemphigoid antigen 230 (BP230), a cytoplasmic component of HD belonging to the plakin family of proteins, is implicated in the attachment of keratin filaments to the plasma membrane. We have recently observed that in a BP180-deficient keratinocyte cell line BP230 is diffusely distributed in the cytoplasm. However, in cells transfected with cDNA for BP180, expression of BP180 affects the cytoplasmic distribution of BP230. BP230 is no longer diffusely distributed in the cytoplasm, but is concentrated in HD-like structures, codistributed with BP180, suggesting a functional interaction between these two proteins. In a yeast-two hybrid (YTH) screen of a keratinocyte cDNA library utilizing the amino-terminal half of BP230 we have identified BP180 as interacting partner. In further YTH assays utilizing deletion mutant forms of BP180 and BP230, it was found that the association between these two proteins involves sequences within the globular head domain of BP230 and the amino-terminal half of the cytoplasmic domain of BP180. Together, the results indicate that the direct interaction of BP230 with BP180 might represent a means by which the recruitment of BP230 into HD is facilitated. These findings further our knowledge of the molecular organization of HD.

002

Vaccination with Mage-3 Peptide-Pulsed Mature, Monocyte-Derived Dendritic Cells Expands Specific CTL and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

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Dendritic cells (DC) are considered a promising approach to induce immunity to cancer. In recent phase I studies, antitumor activity was suggested, but the DC were ill-defined and their ability to expand tumor-reactive T cells was not demonstrated. We explored mature, monocyte-derived DC as adjuvants for resistance to melanoma. The DC were pulsed with Mage-3 A1 peptide + Tetanus Toxoid or tuberculin. 11 far-advanced stage IV HLA-A1 melanoma patients that were progressive despite standard chemo(immuno-)therapy received five DC vaccinations at 14 d intervals. The first three were administered into the skin, 3×10^6 DC each s.c. and i.d., followed by two i.v injections of 6×10^6 and 12×10^6 DC, respectively. Only minor side-effects were observed (local reactions at vaccination sites, fever, and lymph node swellings). Immunity to Tetanus toxoid or tuberculin was boosted in all patients as indicated by antigen specific proliferation assays, IFNγ ELISPOT analyses, as well as strong DTH reactions to DC carrying recall antigen. Considerable expansions, up to 300 fold above preimmune levels, of Mage-3A1 specific CD8+ CTL precursors were induced after the intracutaneous immunizations in eight of 11 patients, and measured in peripheral blood by semiquantitative recall assays. This is significant immunity, as Mage-3A1-reactive T cells have so far been undetectable even after Mage-3A1 peptide vaccination. Curiously, Mage-3A1-as well as recall antigen-specific T cell responses often declined after the i.v., so that the i.v. route may be counterproductive. Objective tumor responses with regression of individual skin, lung and/or liver metastases were evident in six of 11 patients. In another phase I study 8 HLA-A2.1 melanoma patients were vaccinated with Mage-3A2.1 and influenza matrix peptide-loaded DC. Again, induction or expansion of specific CTL including effectors as well as some tumor regressions were observed. Our studies are the first to provide the "proof of principle" that DC vaccination can expand tumor-specific T cells even in advanced cancer patients. This provides a sound basis to rationally optimize DC vaccination strategies in small Phase II trials before clinical efficacy can be determined in large Phase III trials.

003

Expression of SERCA2 Isoforms in Skin, and Mutations in Darier's Disease

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Darier's disease (DD) has recently been shown to be due to mutations in ATP2A2 (SERCA2; Sakuntabhai *et al. Nature Genet* 21:271–277, 1999), a widely expressed ATPase which pumps cytoplasmic calcium into the endoplasmic reticulum for use in calcium signalling. Intriguingly, mice hemizygous for a null mutation in SERCA2 exhibit impaired cardiac performance (Periasamy *et al. J Biol Chem* 274:2556–25, 199). ATP2A2 has 21 exons and two alternatively spliced products, SERCA2a and SERCA2b, which differ only in the C-terminal domain; SERCA2a is the predominant isoform in heart. Although both isoforms were identified in keratinocyte cDNA, expression in skin has not been studied directly.

To examine SERCA2 expression in skin, we have used antibodies recognising the distinct C-terminal domains of SERCA2a and 2b to study normal human skin and cultured cells (gift of Dr F Wuytack, Leuven, Belgium; Wuytack *et al. Biochem J* 257:117–123, 1989). In cultured human keratinocytes and fibroblasts both 2a and 2b-specific antibodies showed clear cytoplasmic immunostaining. On sections of adult skin 2a isoform-specific staining was difficult to distinguish from background, but 2b isoform-specific staining showed clear positivity in epidermal structures including interfollicular epidermis, pilosebaceous units and sweat glands. Little staining was observed in the dermis.

Using SSCP screening and/or direct sequencing, we have identified mutations in over 40 pedigrees of DD, which occur in 19 of 21 exons and include nonsense and splice site mutations predicted to affect expression of both isoforms of SERCA2, in addition to missense mutations which might have specific effects. SERCA2b appears to be the predominant isoform in epidermis, but it is unlikely that the apparent absence of cardiac phenotype in DD is related to the pattern of isoform expression.

004

Tumour Specific Epitopes for Cutaneous T Cell Lymphoma Developed with Randomised Peptide Libraries and Combinatorial Peptide Chemistry

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Mycosis fungoides is the most frequent T cell lymphoma of the skin. In despite of their obvious importance respective tumour antigens have not yet been identified with the exception of a single case where an idiotype derived peptide was found to trigger CTL of the respective patient. The identification of natural antigens is laborious, time-consuming and requires large amounts of tumourmaterial. Here a new approach for the determination of synthetic epitopes for tumour specific CTL with random peptide libraries is presented that can overcome these limitations. Using peptide libraries in OX8 positional scanning format, synthetic epitopes were identified for the CTCL specific CTL clone My-La CTL which is HLA-restricted. The response to these epitopes is comparable to the response to their natural target My-La. Taking these synthetic epitopes, T cells of an HLA-matched patient could be induced *in vitro* and led to the establishment of different cell lines and clones. Some of these lines recognised the peptides as well as the tumour cell line My-La, indicating that they are specific for a naturally expressed tumour antigen. Frequency analysis in 14 HLA-matched patients showed that in more than 70% of the patients $CD8^+$ T cells specific for this synthetically derived epitope are circulating in the blood. These results suggest that there must be a tumour antigen in CTCL, that is common to a wide range of patients. Moreover the identification of synthetic epitopes for tumour specific CTL clones can be used for the development of vaccines for immune therapy of cancer. These vaccines can be applied interindividually. Synthetic epitopes do not necessarily correspond to the natural epitope, but they usually are more potent for stimulation of specific T cells, and can be fine-tuned to increase their potency in therapeutic applications.

005

MIP-3α is Involved in the Constitutive Trafficking of Epidermal Langerhans Cells

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While certain dendritic cells (DC) appear only at inflamed sites, others (e.g., Langerhans cells) traffick through peripheral organs constitutively. We found that $CD1a^+$ Langerhans cell precursors generated *in vitro* from $CD34^+$ stem cells restrictedly and specifically respond to the CC chemokine MIP-3α/LARC. In contrast, non-Langerhans cell DC precursors were attracted by MCP-1 but not by MIP-3α. During their maturation, Langerhans cells loose their migratory responsiveness to MIP-3α and non-Langerhans cell DC do not acquire MIP-3α sensitivity. That MIP-3α is important for Langerhans cell recruitment *in vivo* gains strong support by the observations that (i) freshly isolated epidermal Langerhans cells express the MIP-3α receptor CCR6 both at the mRNA and the protein level; (ii) non-Langerhans cell DC which are absent from normal epidermis as well as cytokine-matured epidermal Langerhans cells lack CCR6 expression; and (iii) MIP-3α is constitutively expressed by keratinocytes. Since mature forms of both Langerhans cells and non-Langerhans cell DC acquire comparable sensitivity for the CCR7 ligand MIP-3β, it appears that chemokines exert DC subtype-restricted migration only at the committed precursor stage. While epidermal Langerhans cells express a restricted set of CC chemokine receptors (i.e., CCR6, CCR7), inflammation-related blood DC stably display a broad chemokine receptor repertoire (CCR1, CCR2, CCR3, CCR5, CCR7) but are CCR6-negative. These findings depict a scenario of two different DC types, one of which (e.g., the Langerhans cells) screens the environment constitutively by following a restricted set of imprinted coding signals and another DC which responds to a wide array of different inflammation-related chemokines and, thus, may be involved in the amplification of ongoing tissue inflammation.

006

$p21^{CIP1}$ Acts as a Positive Regulator of Cyclin B Through Carboxy–Terminal Association with a Novel Protein, CARB

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The cyclin-dependent kinase inhibitor $p21^{CIP1}$ regulates cell cycle progression, DNA replication and DNA repair by binding to specific cellular proteins through distinct amino-and carboxy-terminal protein binding motifs. Thereby p21 acts as a pivotal regulator of epithelial carcinogenesis and differentiation; however, the molecular mechanisms are incompletely understood. Using a yeast two-hybrid strategy we have cloned a novel human gene, *CARB* (*C*IP1-*a*ssociated regulator of cyclin *B*), whose product interacts with the p21 carboxy-terminus *in vitro* and *in vivo*. CARB is expressed abundantly in many human and mouse cell types at the mRNA and protein level. Immunocytochemical analysis with a specific antiserum demonstrates that the CARB protein is associated with the centrosome and mitotic spindle poles. Unexpectedly, the carboxy-terminus of p21 regulates the association between CARB and cyclin B, a key regulator of mitosis. Overexpression of CARB in $p21^{-/-}$ cells, but not in $p21^{+/+}$ cells, results in an accumulation of cells in G_2 followed, ultimately, by cell death. These data identify a novel mechanism which may underlie a positive, rather than negative, activity of p21 in the G_2/M phases of the cell cycle. Moreover, the low frequency of *p21* gene inactivations in human cancers may be due to its requirement for normal cell division.

007

Delayed Wound Healing in Transgenic Mice Overexpressing The Endogenous Angiogenesis Inhibitor Thrombospondin-1 in the Skin

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The matricellular protein thrombospondin-1 (TSP-1) has been identified as a potent endogenous inhibitor of angiogenesis. To characterize the role of TSP-1 during cutaneous wound healing, we investigated TSP-1 mRNA expression in an experimental model of dexamethasone-induced delayed wound healing by RNase protection assays, and we studied wound closure rates of full-thickness wounds in adult transgenic FVB mice overexpressing human TSP-1 under control of the keratin 14 promoter.

During the early phase of experimentally impaired wound healing, TSP-1 mRNA expression was increased in dexamethasone-treated mice, as compared to control mice. K14/TSP-1 transgenic mice were largely healthy, but showed distinct hair growth and vascular alterations in the skin. Full-thickness wounds, induced by 6-mm punch biopsies, healed within 10 d in wildtype mice ($n = 30$ wounds), whereas wound closure was delayed by more than one week in K14/TSP-1 transgenic mice ($n = 30$). Histological and immunohistochemical analyses demonstrated that the formation of granulation tissue was altered in K14/TSP-1 mice, suggesting that the antiangiogenic effect of TSP-1 contributed to the delay in wound closure. No evidence was found for an inhibitory effect of TSP-1 on epidermal keratinocytes, and *in vitro* migration of epidermal keratinocytes obtained from K14/TSP-1 transgenic mice was not impaired, as compared to wildtype keratinocytes.

These findings demonstrate distinct effects of transgenic overexpression of an endogenous angiogenesis inhibitor in the skin and suggest a major role of TSP-1 in the regulation of cutaneous wound healing.

008

Response of Psoriasis to Interleukin-10 is Associated with a Suppression of Cutaneous Th1-Type Inflammation

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Cutaneous lesions in psoriasis are characterized by lymphocytic infiltration and abnormal keratinocyte function. Infiltrating T cells predominantly belong to the T helper type 1 (Th1) subset with skin lesions containing high levels of IL-2, IFNγ and TNFα. IL-10 was shown to play an important role in the endogenous and exogenous suppression of cutaneous Th1-type responses. To investigate the biological effects of IL-10 in psoriasis 14 patients with moderate-to-severe disease were treated with systemic rhIL-10 over 6 wk. Biopsies obtained at baseline, after 3 d and at the end of treatment were analysed by immunohistochemistry and semiquantitative RT-PCR. Marked clinical improvement was observed in 10 patients (PASI decrease $> 50\%$), four patients had moderate improvement (PASI decrease $\leq 50\%$). The clinical response was associated with a marked decrease of the T cell infiltrate. In the high clearing group, the median epidermal T cell count ($CD3^+$ per mm surface) decreased from 84 (range: 40–136) before to 28 (8–92) after IL-10 therapy ($p < 0.01$), and the median T cell count in the upper corium ($CD3^+$ per mm^2) from 317 (155 to 460) to 141 (84–421; $p < 0.01$). Parallel to the reduction of infiltrating T cells the expression of Th1-type cytokines IFNγ and TNFα was also markedly decreased. A significant correlation between the PASI decrease and the decrease of cutaneous IFNγ ($r = 0.56$; $p < 0.05$) and TNFα levels ($r = 0.64$; $p < 0.05$) supported an association between clinical improvement and suppression of Th1-type cytokine production. There was a strong early suppression of cytokines involved in T cell activation and Th1-type differentiation such as IL-1β, IL-2 and IL-12p40 after 3 d of treatment, preceding the full effect on IFNγ and TNFα production. Individual variations in the downregulation of TNFα by IL-10 were found to be associated with polymorphisms in the TNFα promoter in that carriers of variant alleles appeared largely resistant towards IL-10 mediated suppression of skin TNFα levels. Our findings suggest that antipsoriatic activity of IL-10 is based on the reduction of cutaneous inflammation and local production of Th1-type cytokines and that genetic factors might account for individual differences in the clinical response.

009

A Glycine Substitution in the First EGF-Like Repeat of the Laminin β3 Chain Results in a Mild Form of Junctional Epidemolysis Bullosa

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Junctional epidermolysis bullosa (JEB) is an inherited mechanobullous disorder characterized by formation of blisters and erosions of the skin and mucous membranes. In the blisters, the cleavage plane lies within the lamina lucida of the dermal–epidermal junction. We report the case of a patient, product of a consanguineous union, presenting with mild JEB characterized by head and acral blisters, and urethral stenosis consequent to wound healing. Nail dystrophy and dysplastic teeth were also observed. Electron microscopy detected mature hemidesmosomes. Immunofluorescence analysis of the patient's skin revealed strongly reduced reactivity of the basement membrane zone to the antibodies directed against laminin-5 and collagen XVII. Conversely, the basal keratinocytes presented a strong cytoplasmic staining. Immunoprecipitation and western analysis of the proband's keratinocytes revealed a cytoplasmic localization of laminin-5 and BP180. Transfection of the proband's keratinocytes with curative transgenes demonstrated that a wild type laminin β3 cDNA restored the secretion of laminin-5 and collagen XVII *in vitro*. A search for mutation in the gene LAMB3 identified a homozygous mutation ($761G \rightarrow A$) that converts a glycine into aspartic acid (G254D) in the first EGF-like domain of the laminin β3 chain short arm. Transfection of a mutated laminin β3 cDNA bearing the $761G \rightarrow A$ substitution in β3-null keratinocytes restored synthesis of laminin-5 molecules that were retained in the cytoplasm, as observed in the proband's keratinocytes.

These results suggest a role of the first EGF-like domain of the β3 short arm in the secretion of laminin-5. They suggest that the presence of laminin-5 in the extracellular matrix determine the correct positioning of BP180 in the basement membrane.

010

Generation of Hybrid Cells by Fusion of Allogeneic Dendritic Cells with Autologous Tumor Cells and First Clinical use in Patients with Metastatic Melanoma

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Hybrid cell vaccination is a cancer immunotherapy that aims at recruitment of T cell help for induction of tumor-specific cytolytic immunity. Epitope linkage has been shown to be prerequisite for productive T-T cell collaboration, i.e., cytolytic precursor and helper T cells have to be activated simultaneously by the same antigen presenting cell displaying epitopes for both T cell types on the corresponding MHC class I and class II molecules. The vaccine generated by fusion of the patients' tumor cells with allo MHC II-bearing cells combines the tumour's antigenicity with the immunogenicity of allogeneic MHC-molecules. We have previously shown that hybrid cell vaccination of allogeneic B cells fused with tumor cells is a well tolerated treatment which shows considerable clinical efficacy in patients with metastatic melanoma.

Now we asked whether dendritic cells could be used for the same purpose. DCs were generated by adding GM-CSF (50 ng per ml) and IL-4 (1000 μ per ml) to monocytes obtained after adherence of PBMC collected by leukapheresis of healthy donors. Single cell suspensions of autologous melanoma metastasis were mixed with DCs at a ratio of 1:3 and fused by electrofusion. Fused cells were detected by double staining of hybrid cells with dendritic cell and tumor cell markers. The hybrid cells expressed high levels of class I and II, ICAM-1, B7-1 and B7-2. For evaluating the *in vivo* efficacy we have subcutaneously injected three melanoma patients with an individually prepared and irradiated hybrid cell vaccine on a monthly basis. While this treatment was well tolerated, 2/3 patients showed an increased DTH reaction to autologous melanoma cells. One patient showed a complete response, one had a partial response and the third showed stabilization of large abdominal tumor masses. Immuno-histological analysis of a responding metastases showed a complete replacement of tumor cells by a dense inflammatory infiltrate and macrophages. Taken together, the system of hybrid cell vaccination has been successfully expanded to the use of dendritic cells as part of the vaccine.

011

Peptides and Naked DNA Encoding a Sequence for T Cell Receptor α Chain: A Novel Tool for the Treatment of Inflammatory Skin Reactions

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Recently we could demonstrate that a core peptide (CP) encoding a sequence for the transmembrane region of the T cell receptor α chain (TCR-α) inhibits T-cell function by preventing functional assembly of the T cell receptor. In an allogeneic MLR proliferation of $CD4^+$ as well as $CD8^+$ T cells was reduced by preincubation with CP but not control peptide. Simultaneous addition of an anti-CD3 mAb completely restored proliferation demonstrating the specificity of the effect for the T cell receptor assembly. In a model of murine contact sensitivity application of the peptide reduced the elicitation of an ear swelling response following application of a contact allergen in sensitized animals by 50%. To achieve a prolonged effect of the peptide we furthermore used plasmids containing the core sequence of the T cell receptor-α (TCR-α) chain. The T cell receptor transmembrane plasmids pTCRTMB and pTCRTMBA were constructed by cloning a 27 bp fragment into a secretory plasmid vector (Invitrogen, San Diego, CA). The negative control plasmid pTCRTMBA contains the same 27 bp fragment in antisense orientation. To assess functional activity of the DNA, HaCat cells were transfected with the plasmids by lipofection. $CD4^+$ as well as $CD8^+$ T cells were preincubated with the supernatant of transfected HaCat cells. In an allogeneic MLR using dendritic cells as APC proliferation of $CD4^+$ or $CD8^+$ T cells was reduced by preincubation with supernatant of pTCRTMB transfected HaCat cells by 70%, but not by the negative control plasmid (pTCRTMBA). Incubation with an anti-CD3 mAb abolished this inhibitory effect. When naked DNA encoding the peptide sequence was injected into skin prior to application of a contact allergen to sensitized animals local immunosuppression was also observed. To investigate the effect of the peptide in humans, patients with psoriasis, atopic eczema, lichen planus or contact dermatitis were treated with the TCR-α peptide or control peptide. All patients except for one reported about a marked improvement or cure of the disease following application of the TCR-α peptide, but not controls. These data indicate that TCR-α peptide treatment might be a proper treatment for human T-cell-mediated dermatoses substituting for corticosteroids.

012

Continuous Intralymphatic Application of MHC/HLA Class I Binding Antigenic Peptides as a Novel Method to Induce Strong Cytotoxic T Cell Responses against Melanoma

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Our studies investigated the parameters which determine the immunogenicity of a class 1 MHC binding antigenic peptide. We demonstrate that the route and duration of administration represent crucial parameters determining the immunogenicity of such a vaccine. Regarding the route of administration, direct injection into a lymph node or into the spleen is found to be 10^6 times (!) more efficient than subcutaneous or dermal injection and around 10^4 times more efficient than intravenous injection. Regarding the duration of administration we find that only continuous peptide delivery, but not injection as a single bolus, does efficiently induce $CD8^+$ cytotoxic T cell responses. The cytotoxic T cell response induced by continuous peptide administration directly into a lymphatic organ is short lived. It provides protection against tumor challenge merely for 2–3 d.

In a clinical trial with stage IV melanoma patients we therefore place a catheter directly into the inguinal lymph node under ultrasound control. Over the time period of 1 wk the lymph node is then perfused with class 1 HLA-binding melanoma-specific peptides by the use of a portable microperfusor. Cycles of immunization are repeated with weekly intervals. This way of immunotherapy rapidly generates strong peptide specific T cell responses and tumor regression can be observed.

013

MHC Class I Binding and Immunostimulatory Properties of Peptides Derived from T Cell Receptors of CTCL Cells

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A candidate tumor antigen in cutaneous T cell lymphoma (CTCL) is the clonotypic T cell receptor (TCR). However, limited information exists concerning the MHC class I binding ability and, more importantly, the immunogenicity of human TCR-derived peptides. In order to generate essential tools to address these issues, we cloned and sequenced cDNAs encoding the idiotypic TCRα and β chains of $CD4^+$ CTCL cells. Selective TCRVα/β usage of isolated CTCL cells was confirmed by a V region-specific PCR assay and the clonality of TCR amplicons was assessed by sequence comparisons of multiple cDNA subclones. The deduced full-length TCR amino acid sequences from an $HLA\text{-}A2^+$ patient were subjected to an anchor position-based motif search for nonameric HLA-A2 peptide ligands. Peptides located within the V-, CDR3-, and C-regions from either TCR subunit were selected based on their $T_{1/2}$ score of > 10. Binding of these peptides was tested on $HLA\text{-}A2^+$ TAP-deficient T2 cells. T2 cells were exposed to TCR-derived peptides in the presence of exogenous β2m and the ability of peptides to reconstitute conformationally intact HLA-A2 was monitored using mAb W6/32. Peptides VIFGPTSL (CDR3α) and ILWLQPDWV (Vα21) reconstituted MHC class I complexes in a magnitude comparable to that achieved by a high-avidity HLA-A2 binding, hepatitis B virus-derived control peptide (K_d = 3.3×10^{-9} M). In binding competition assays, the HBV control and the Vα21 peptides displayed similar affinities for HLA-A2 while the CDR3α peptide bound HLA-A2 even more efficiently than did the HBV peptide. The four remaining TCR (Vβ17, Cβ1, CDR3β, Cα) peptides also reconstituted W6/32 immunoreactivity and competed with HBV peptide binding although less efficiently than the Vα21- and CDR3α-derived moieties. To see whether TCR-derived peptides are presented in immunologically relevant fashion, T lymphocytes from $HLA\text{-}A2^+$ donors were repeatedly stimulated with Vα21 and CDR3α peptide-loaded dendritic cells. T cell lines generated were tested for cytotoxicity against peptide-pulsed T2 cells. T cell blasts efficiently lysed Vα21 but not CDR3α or gp100/pmel17 control peptide-loaded targets. Immunodepletion and mAb-based blocking experiments further revealed that this Vα21 peptide-specific cytotoxic activity is mediated by $CD8^+$ rather than by $CD4^+$ T cells. Thus, it appears that clonotypic TCRs of CTCL cells can contain several high-affinity MHC class I binding peptides and that at least for certain epitopes peptide-specific CTL precursors exist. Provided that the proteasomal machinery of CTCL clones allows the generation and presentation of TCR-derived peptides *in vivo*, some of these moieties may well serve as relevant immunogens for vaccination strategies aiming at inducing protective antitumor immunity.

014

A Non-HLA Positional Candidate Gene for Psoriasis Vulgaris Within the MHC

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Genome wide linkage analyses have identified a replicated psoriasis susceptibility locus in a 12-cM interval on chromosome 6p21.3 between markers D6S426 and D6S276, containing the MHC. To localise susceptibility gene(s), family studies for linkage disequilibrium and haplotype were performed on 118 affected sibling pairs.

Fourteen polymorphic markers were genotyped including HLA-B and -C, across the MHC. Significant linkage and allelic association was identified for a 285 Kb region defined by markers tn62 ($p = 1.0\times10^{-7}$), HLA-B ($p = 4\times10^{-7}$) and HLA-C ($p = 2.7\times10^{-9}$). The commonest haplotype included HLA-B*5701 and HLA-Cw*0602, the next most common haplotype included Cw*0602 but not B*5701. Within this region is the "S" gene, which encodes corneodesmosin, a late differentiation epidermal protein. Its genomic position 160 Kb telomeric of HLA-C suggests a potential role in psoriasis susceptibility. Parent-offspring trios ($n = 152$) from 99 families were genotyped for S gene bi-allelic polymorphisms at positions +619, +1240 and +1243, inherited in cis. Allelic transmission to affected individuals was compared with nontransmission (transmission disequilibrium test TDT). S gene allele 5 showed significant evidence of linkage and disease association ($p < 0.000003$). 38% of allele 5 transmissions from heterozygote parents were independent of the HLA-Cw6 haplotype, indicating a role independent of HLA-C, for the S gene in psoriasis susceptibility. Corneodesmosin functions in differention and adhesion in the stratum granulosum (SG) and corneum (SC), in psoriasis these functions are altered and SG is lost. Our data thus suggest the S gene is potentially a better candidate for psoriasis susceptibility than HLA-C.

015

Comparison of cDNA Expression Between a High- and a Low-Invasive Melanoma Cell Clone Using an Microarray System

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Previously, we have selected two cell subpopulations derived from the melanoma cell line Mel Im. The two subpopulations were isolated using a Boyden Chamber system to select for highly (cell clone hi) and weakly invasive cells (cell clone si). After several selection steps we obtained cell clones which differed 5-fold in their invasive potential. Further evaluation also showed changes in their potential to metastasis in nude mice. To analyse differentiell expression of mRNAs comparing these two cell clones we used a cDNA microarray system (Clontech).

In total we detected 23 cDNAs which were differently regulated. 10 of these cDNAs are upregulated in the highly invasive cell clone, 13 are downregulated.

As expected, both cell clones were HLA I and p16 negative. Many cytoskeletal proteins as desmin, vimentin and several cytokeratins are strongly downregulated in the high invasive cell clone. This finding correlates with our previously published data that γ-actin is downregulated in the highly invasive cells. cDNA expression of many proteins involved in positive regulation of apoptosis is downregulated in the more invasive cell clone hi, further slight upregulation of matrix metalloproteinases (MMP-9, MMP-16) and EMMPRIN, an activator of MMPs, was found. Interestingly, we found downregulation of interferon receptors in the high invasive cells. Concerning growth regulatory genes, upregulation of some growth factor cDNAs, ephrin type B receptor 1 and cyclin C, but downregulation of inhibitory proteins like p19 are found in the highly invasive cells.

016

Connexin Mutations in Skin Disease and Deafness: Genotype *Versus* Phenotype

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Mutations in gap junction proteins can underlie both dominant and recessive forms of sensorineural deafness and also autosomal dominant skin disease. Although Connexin 26 is expressed in the epithelial cells of the cochlea and the suprabasal epidermis (as is Connexin 31), autosomal recessive Cx26 mutations result only in deafness (indeed are a major cause of genetic sensorineural deafness). In this study we have sequenced Cx26 and Cx31 in families with autosomal dominant skin disease in which deafness may or may not be segregating in order to explore if there were any correlations with site of mutation and the development of skin disease or deafness or indeed both phenotypes. We describe the identification of mutations in Cx26 and Cx31 which underlie two types of skin disease, erythrokeratoderma variablis (R43P in Cx31) and a Vohwinkel's pattern of palmoplantar keratoderma (D66H in Cx26). The mutations are likely to disrupt voltage gating and result in poor connexon-connexon formation leading to a breakdown in cell-cell communication. For the D66H skin disease associated mutation, some carriers had normal hearing, some had high frequency hearing loss and others were profoundly deaf. This could be explained by partial penetrance of the Cx26 mutation with regard to hearing status (as fully penetrant for the skin phenotype). However, we demonstrate that the severity of the skin disease and the hearing loss (either high frequency or profound deafness) in the carriers of the D66H mutation is associated with additional modifying mutations in Cx26 and Cx31.

017

The Transcriptional Activity of E2F2 and E2F4, Required for Cell Proliferation, is Deregulated in Melanomas

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The retinoblastoma tumor suppressor family of proteins (pRb, p107, p130, referred to as pocket proteins) are critical regulators of cell cycle progression. The hypo- or un-phosphorylated forms of the pocket proteins arrest proliferation by binding to E2F transcription factors (E2F1-E2F5) and suppressing gene expression. Phosphorylation of pocket proteins by cyclin-dependent kinases (CDKs) disrupts these complexes and releases the transcriptional activity of E2Fs. Since Rb is commonly inactivated in melanoma cells by hyperphosphorylation or decreased expression, we determined which of the E2F family members are active in normal *versus* malignant melanocytes. Using gel shift analyses, we identified E2F2 and E2F4 as the two transcription factors active in proliferating normal melanocytes, melanoma cells and freshly isolated tumors. In normal melanocytes, E2F2 and E2F4 were present at low levels and in complex with pocket proteins, unless the cells were stimulated by synergistic growth factors. In contrast, E2F2 and E2F4, and in some cases E2F5, were highly abundant and active in freshly isolated tumor cells and in several melanoma cell lines grown in the absence of melanocyte-growth factors. We suggest that the continuous inactivation of pocket proteins in melanoma cells by constitutively active CDKs allows the accumulation of unregulated E2F2 and E2F4 activity. The transcriptional activity of these factors induces the production of proteins required for cell cycle progression, releasing the cells from external constraints and supporting autonomous cell growth characteristics to melanomas.

018

Rescue of Pigmentation Phenotype in *ee* Mice Transgenic for Murine and Human Wild Type Melanocortin 1 Receptor Genes Demonstrates that this Receptor Controls both Interfollicular and Follicular Pigmentation

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Individuals with red hair and fair skin frequently contain variants of the human melanocortin 1 receptor (*MC1R*) gene, but subjects with fair skin without red hair may or may not contain *MC1R* variants, questioning the contribution of *MC1R* to interfollicular pigmentation and human skin type. To investigate this further, we have utilised *ee* mice, which are functionally null at the murine *mc1r* locus resulting in a yellow coat colour, and have generated mice transgenic for wild type murine *mc1r* and human *MC1R* genes. Screening of a bacterial artificial chromosome (BAC) library allowed isolation of a 120 Kb BAC containing the entire murine *mc1r* gene. Pronuclear injection of the unmodified BAC gave 13 transgenic lines, with rescue of the pigmentation phenotype in eight lines (alteration in coat colour from yellow to agouti, and in tail/ear skin colour from yellow to brown). Homologous recombination of the BAC in *E. coli* allowed substitution of the murine *mc1r* coding region by human wild type *MC1R* coding sequence coupled to an IRES/lac Z. Generation of four lines transgenic for this modified BAC resulted in a similar alteration in coat and skin colour from yellow to agouti and brown, respectively, in two lines. The results demonstrate that both the human and murine melanocortin 1 receptors control interfollicular in addition to follicular pigmentation *in vivo*.

019

Dendritic Cells Cross-Present Tumor Associated Antigen to Autologous $CD8^+$ T Cells after the Uptake of Apoptotic Melanoma Cells

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It has recently been demonstrated that dendritic cells (DC) take up apoptotic cells via the vitronectin receptor αvβ3 and are capable of presenting influenza antigen from flu-infected apoptotic monocytes in a MHC-I context. We wanted to demonstrate the existence of this cross-presentation mechanism for tumor antigens. We induced apoptosis in an HLA-A2 negative melanoma cell line (MEL397) with UVB-irradiation. Apoptotic MEL397 cells were cocultured with immature DC generated from PBMC of HLA-A2 positive healthy donors. Apoptotic tumor cells labelled with life dye (PKH26) were taken up rapidly and efficiently by DCs, but did not induce DC maturation. We thus matured DCs after the phagocytosis by using a cytokine cocktail (IL-6, IL-1β, TNF-α and PGE-2). The repetitive restimulation of autologous $CD8^+$ cells with these DCs (cross-presenting DC) yielded a substantial induction of T cell proliferation in the presence of 40 iU per ml of IL-2. To evaluate the specificity of these CTLs we used synthetic 9-mer peptides representing the HLA-A2 immunodominant sequences of tumor associated antigens (TAA). CTLs synthesised TNF-α when stimulated with peptides of TAAs expressed by MEL397 (MelanA/MART1 and MAGE-3) but not when stimulated with tyrosinase, which is not expressed by MEL397. After 2–3 cycles of restimulation, CTLs were able to lyse TAP deficient T2 target cells pulsed with MelanA/MART1 or MAGE-3, but not tyrosinase, in ^{51}Cr release assays. When directly compared with CTLs generated with peptide pulsed DCs, cross-primed CTLs were less effective in killing peptide pulsed T2 cells, indicating a rather weak expression of TAAs on the surface of cross-presenting DCs. Furthermore, CTLs generated with peptide pulsed DC were not able to lyse allogeneic cross-presenting DC. When the HLA-A2 positive melanoma cell line (MEL 526) was used as target cell, lysis was more efficient by cross-primed CTLs compared to CTLs generated with peptide pulsed DCs. This might indicate the cumulative activity of CTLs with different specificity resulting from cross-priming. We conclude that TAAs are cross-presented after the uptake of apoptotic tumor cells. This knowledge might enable the development of new DC vaccine strategies using apoptotic autologous or allogeneic tumor cells.

020

Melanoma-Specific CD4+ T Cells Recognize HLA-DR-Restricted Melan-A/MART-1 Antigen: Implications for Vaccine Strategies

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Many MHC Class-I melanoma antigens recognized by CD8+ T cells have been reported but few MHC Class II-restricted epitopes recognized by CD4+ T cells have been identified so far. CD4+ T cells play critical roles in the induction and maintenance of antitumor responses. However the real benefit that may result from induction of CD4+ T cells in human antitumor immunity remains yet to be assessed. We focused our study on the CD4+ T cell responses in long-lived patients with melanoma. In one of HLA-A2+/DR+ long-lived patients, we obtained CTL clones from peripheral blood lymphocytes (PBL) directed against the HLA-A2 restricted Melan-A/MART-$1_{27–35}$ epitope and therefore we specifically focused upon the anti-Melan-A/MART-1 CD4+ T cell response. We have used Melan-A/MART-1 DR-binding peptides predicted by a peptide-binding algorithm to generate specific CD4+ T cells. These CD4+ T cells specifically produce IFN-γ (in IFN-γ ELISPOT assays) and lyse not only DR+-antigen presenting cells (APC) pulsed with one of the Melan-A/MART-1 peptides but also the autologous tumor cell line, expressing the Melan-A/MART-1 gene. Using biochemical techniques, the endogenous peptides were eluted from the HLA-DR molecules expressed by the melanoma cells and tested for bioactivity in IFN-γ ELISPOT assays in the presence of the specific CD4+ T cells. We demonstrate a Melan-A/MART-1 peptide that is naturally processed and presented at the surface of these melanoma cells. IFN-γ ELISPOT assays were performed using CD8+ and CD4+ T cells isolated directly from PBMC at various time points during the course of disease. Strong anti-Melan-A/MART-$1_{27–35}$ CD8+ T cell responses and immune reactivity against the DR-Melan-A/MART-1 epitope were observed. No reactivity was observed in three normal donors.
In conclusion, we have identified a new DR-restricted Melan-A/MART-1epitope. The demonstration of this new HLA-DR-restricted epitope adds to the list of HLA-A2-restricted and HLA-A3-restricted epitopes already identified within the Melan-A/MART-1 molecule. Thus with its frequent and homogeneous expression in melanoma specimens and its capacity to generate both CD4+ and CD8+ T cell responses, Melan-A/MART-1 may provide optimal immunization against melanoma if used as a multiepitope peptide vaccine.

021

GM-CSF-Based Melanoma Vaccines: Dendritic Cells Represent the Critical Antigen Presenting Cells (APC)

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It is known that the s.c. administration of GM-CSF-transfected cancer cells protects experimental animals against the growth of wild-type cancer cells through a T cell-mediated immune response. The goal of the present study was the delineation of the mode of antigen presentation by identifying the relevant APC.
In a first set of experiments, we used a contact hypersensitivity system (CHS), a classical model for T cell-mediated immunity. GM-CSF-secreting M3 melanoma cells (M3-GM; H-2d) or bone marrow-derived dendritic cells (BMDC) were haptenized with TNCB and then s.c. injected into either syngeneic or allogeneic hosts. Haptenized BMDC (H-2d) elicited a CHS response only in syngeneic (H-2d) recipients, compatible with the expected mode of direct antigen presentation. By contrast, haptenized M3-GM cells were able to induce an ear swelling response in syngeneic as well as in allogeneic mice, arguing for a critical role of host APC. To identify these APC, we introduced the βgalactosidase gene into M3-GM cells and used βgal-specific CTL to probe draining lymph node cells for the presence of antigen-bearing cells. In a first step, we could demonstrate that M3-GM-βgal cells induce βgal-specific CTL when s.c. injected into DBA/2 mice. The evaluation of lymph nodes draining M3-GM-βgal injection sites for βgal protein-expressing cells yielded negative results. We then assessed these lymph node cells for the presence of βgal epitope-bearing cells using a specific T cell clone. Results obtained showed that lymph nodes draining M3-GM-βgal-, but not those of M3-GM injection sites contain cells able to stimulate the βgal-specific CTL-clone to produce IFN-γ. Time kinetic studies revealed that these cells appear by d3, peak by d4–5 and are detectable until d6. Employing positive selection techniques (MACS- and FACS-sorting), we found that the antigen presenting activity resides within the CD11c-positive lymph node cell population.
Our data demonstrate for the first time that DC represent the critical APC in the immune response to GM-CSF-secreting melanoma cells and have important implications for the development of new and more effective vaccination strategies.

022

$CD14^+$ Human Hemopoietic Precursor Cells Develop into Langerhans Cells upon Transforming Growth Factor β1 Signaling

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Langerhans cells are $CD1a^+$ E-cadherin (E-cad)$^+$ Birbeck granule (BG)$^+$ but $CD11b^-CD36^-$ $FXIIIa^-$ members of the dendritic cell (DC) family. Evidence holds that Langerhans cells originate from a pool of $CD1a^+/CD14^-$ rather than $CD14^+/CD1a^-$ progenitors arising in the culture of $CD34^+$ stem cells upon stimulation with GM-CSF/TNF-α. $CD14^+/CD1a^-$ progenitors, on the other hand, can give rise to a separate DC type characterized by its $CD1a^+CD11b^+CD36^+FXIIIa^+$E-$cad^-BG^-$ phenotype (non-Langerhans cell DC). While GM-CSF/TNF-α are important for both Langerhans cells and non-Langerhans cell DC differentiation, transforming growth factor-β1 (TGF-β1) rather selectively promotes Langerhans cell development *in vitro* and *in vivo*. However, the mechanisms involved in this process and the nature of TGF-β1-responsive Langerhans cell precursors (LC_p) are not well understood. Here we show that $CD14^+$ precursors in the presence but not in the absence of TGF-β1 give rise to a cellular progeny that fulfills all major criteria of Langerhans cells. In contrast, Langerhans cell development from $CD1a^+$ progenitors was TGF-β1-independent. We also found that $CD14^+$ precursor subsets exist which strikingly differ in their ability to undergo Langerhans cell differentiation. In the presence of TGF-β1, $CD14^+CD11b^-$ cells differentiated into Langerhans cells while their $CD11b^+$ counterparts acquired non-Langerhans cell DC features only. High doubling rates of precursor cells entering the $CD14^+$ LC_p pathway and, as a result, a larger pool of TGF-β1-responsive $CD14^+$ LC_p than of $CD1a^+$ LC_p adds to the importance of TGF-β1 for Langerhans cell development. Since $CD14^+CD11b^-$ precursors are multipotent cells which can enter Langerhans cells or monocyte differentiation, it is suggested that these cells, if present at the tissue level, endow a given organ with the property to rapidly generate functionally diverse cell types in response to the local cytokine milieu.

023

Allergic Contact Dermatitis in Mice Deficient in p75 Nerve Growth Factor Receptors, Neurokinin 1 Receptors or Neutral Endopeptidase

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Sensory nerves that innervate the skin directly contact epidermal and dermal cells and modulate functions of skin and immune cells by the release of neuropeptides such as substance P (SP) or neurokinin A (NKA). NKA and in particular SP are capable of modulating neurogenic inflammation by inducing plasma extravasation and vasodilatation, upregulating cytokines, chemokines and cellular adhesion molecules and by modulating leukocyte effector functions via the activation of specific neurokinin receptors (NK-R). The effects of SP are controlled by the local concentration of SP, the cell-specific expression of its high-affinity NK-1R and of the principal SP-degrading peptidase neutral endopeptidase (NEP). Little is known regarding the relative contributions of SP, NK-1R, NK-2R and NEP and the importance of the cutaneous sensory nervous system in skin inflammation. Our study tests the hypothesis that alterations or defects in the cutaneous nervous system will result in an impaired cutaneous inflammatory response *in vivo*. We analyzed the inflammatory response to dinitrofluorobenzene (DNFB) in a murine model for allergic contact dermatits (ACD) utilizing genetically engineered mice with homologous deletions of either the p75 nerve growth factor receptor gene (NGR-R−/−), which are characterized by a reduced density of cutaneous sensory nerves, the NK-1R (NK-1R−/−) or the NEP gene (NEP−/−) in comparisson to normal wild type (+/+) mice. The allergic ear swelling response to DNFB in sensitized NGF-R (−/−) or NK-1R (−/−) mice was significantly blunted to 50%/40%, respectively, of that observed in the corresponding (+/+) animals. Histologically, NGF-R (−/−) as well as NK-1R (−/−) mice had less edema and a significantly reduced number of infiltrating leukocytes to 59% and 51% of that in NGF-R (+/+) and NK-1R (−/−) animals, respectively, during ACD. In contrast, the ACD response in NK-1R (−/−) and (+/+) wt mice treated with a selective and potent antagonist of the NKA-specific NK-2R was significantly enhanced. In addition, we also observed an augmented cutaneous inflammatory response to DNFB in NEP (−/−) mice compared to (+/+) animals as determined by plasma extravasation, which was quantitated by Evans blue leakage from dermal vessels, ear swelling response and histological examination. These results indicate that an intact cutaneous nervous system is required for a full inflammatory response to allergens. SP, NK-1R, NK-2R and NEP have an important role in the regulation of cutaneous inflammatory responses.

024

Keratinocytes Take-Up Naked Plasmid DNA: Evidence for DNA Binding Proteins in Keratinocyte Membranes

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Epidermal keratinocytes possess the ability to internalize naked plasmid DNA and express reporter genes when plasmid DNA is injected into the dermis *in vivo*. The mechanism by which keratinocytes internalize plasmid DNA is unknown, but a protein-mediated uptake has been suggested. To further analyse the DNA-binding of keratinocyte membrane proteins, a protein fraction was prepared from lysates of human breast epidermis and porcine mucosa and subjected to DNA-binding experiments. Double-stranded DNA (linearized pcDNA3, Invitrogen and sonicated calf thymus DNA, Gibco) was used for hybridization in South-Western technique and Digoxigenin-labelled single-stranded 20- or 40-mer oligonucleotides in gel shift assays. We identified in South-Western experiments three candidate DNA-binding proteins. Competition analysis of DNA binding by unlabelled DNA revealed a high affinity for DNA-binding. In 2-D gels, two DNA-binding proteins could be identified as moesin and ezrin by mass spectrometry. In gel shift assays using various oligonucleotides (single-and double-stranded) two DNA-binding proteins were found in the epidermis and three in mucosal tissue that were different from histones. The binding was not sequence-specific.
These observations suggest the involvement of proteins in keratinocyte binding and uptake of single-stranded and double-stranded DNA of various sizes. However, the exact mechanism of transmembrane transport remains elusive since moesin and ezrin are localized on the inner side of plasma membranes, but are known to be associated with membrane receptors.

025

Targeting of Membrane-Type-1 Matrix Metalloproteinase Activity in Melanoma Cell Invasion and Inactivation by Cleavage of 20-kDa N-terminal Domain

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Tumor invasion is dependent on tightly regulated proteolysis. Membrane-type matrix metalloproteinase (MT1-MMP) is a cell surface receptor and activator for gelatinase A. Relocalization of MT1-MMP and gelatinase A activity to special cell surface sites has been found to induce melanoma cell invasion *in vitro*. The roles of cytoplasmic domain of MT1-MMP on MT1-MMP expression and cell invasion were analyzed in human Bowes melanoma cells by site-directed mutagenesis. Gelatinase A was activated in all cells expressing at their surfaces wild-type or MT1-MMP forms containing a deletion (567–582, 573–582, 577–582) or point mutation (T567A, Y573A, S577A) in the cytoplasmic domain. Cell invasion *in vitro* through basement membrane matrices was induced by expression of wild type and truncated MT1-MMP lacking amino acids 577–582. Longer C-terminal deletions decreased the invasiveness. N-terminal processing of MT1-MMP to an inactive 43 kDa form correlates with gelatinase A activation in fibrosarcoma cells (Lehti *et al.*, *Biochem J* 334:345, 1998). Immunoblotting analyses indicated that MT1-MMP was processed to the 43 kDa cell surface form also in melanoma cells in association with the appearance of a previously unknown soluble ~20 kDa cleavage product. The cytoplasmic domain of MT1-MMP has an important role in the regulation of cell invasion, evidently by targeting MT1-MMP via generation of foci of gelatinase A activity. Inactivation of MT1-MMP by cleavage of N-terminal fragment containing the active site is a rapid step of negative regulation required for tightly controlled proteolysis.

026

Role of CD18 Deficiency in Delayed Tissue Repair

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Patients with mutations in the CD18 gene (β_2-integrins) suffer among other symptoms from impaired wound healing. Using CD18 (β_2-integrin chain) deficient mice, we addressed here the questions whether (1) wound healing is also impaired in CD18 null mice and if so, whether (2) the delay in wound healing is due to subclinical or overt infection or to mechanisms still unknown. Independent of broad spectrum antibiotics (sulfamethoxazol and trimetoprim), there is a highly significant ($p < 0.0001$) inhibition of closure of standardized full thickness wounds expressed as percentage of original wound size in CD18 null mice being most prominent at day 7 post wounding. In addition, microbial analysis of swabs from wounds and wound tissue did not reveal significant differences in the number of bacterial colonies thus providing evidence that infection does not play a major role in delayed wound healing in CD18 null mice. Virtually no neutrophils are found in mechanically and chemically induced wounds suggesting that the CD18 molecule constitutes the exclusive pathway for neutrophil emigration into the skin. By contrast, no difference was found in the number of eggressed monocytes which during emigration most likely depend on alternate molecules such as VCAM-1. Interestingly, the ratio of hydroxyproline to total protein as a measure of collagen synthesis in implanted sponges was found to be significantly reduced ($p < 0.0001$) over a time course of 60 d postwounding in CD18 null mice compared to wild type mice. Representative histology on sponges revealed that the inflammatory phase of wound healing is substantially prolonged in CD18 null mice. In addition, the cytokine profile, particularly of proinflammatory cytokines with matrix-degrading properties such as interleukin-1, interleukin-6 and vascular endothelial growth factor is up to 15-fold increased in wound tissue or bone marrow-derived macrophages upon different stimuli related to tissue repair (LPS, hypoxia, lactate). Taken together, these data unequivocally show that CD18 plays a major role in controlling inflammatory responses and – if absent – leads to a significant delay in complex processes such as tissue repair.

027

A Role for CD40–CD40 Ligand Interactions in the Generation of Type 1 Cytokine Responses in Human Leprosy

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The interaction of CD40 ligand (CD40L) expressed by activated T cells with CD40 on macrophages has been shown to be a potent stimulus for IL-12 production. The expression and interaction of these two molecules were investigated in human infectious disease, using leprosy as a model. CD40 and CD40L mRNA and surface protein expression were found to be predominant in skin lesions of resistant tuberculoid patients as compared to the highly susceptible lepromatous group. IL-12 release from peripheral blood mononuclear cells (PBMC) of tuberculoid patients stimulated with *M. leprae* was partially inhibited by monoclonal antibodies to CD40 or CD40L, correlating with antigen-induced upregulation of CD40L on T cells. Cognate recognition of *M. leprae* antigen by a T-cell clone derived from a tuberculoid lesion in the context of monocyte antigen presenting cells resulted in CD40L–CD40 dependent production of IL-12. In contrast, *M. leprae* induced IL-12 production by PBMC from lepromatous patients was not dependent on CD40L-CD40 ligation, nor was CD40L upregulated by *M. leprae*. Furthermore, IL-10, a cytokine predominant in lepromatous lesions, blocked the IFN-gamma upregulation of CD40 on monocytes. These data suggest that CD40L–CD40 interactions play a prominent role in augmenting Th1 cytokine responses in human infectious disease and that the activation of this pathway is dependent upon the local T-cell repertoire and cytokine pattern.

028

Induction of Th2 Cytokines by Allergen-Pulsed Dendritic Cells in Atopic Individuals and its Shift Towards Th1 by Collagen Type I

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Due to their production of bioactive IL-12 mature dendritic cells (DC) preferentially induce a Th1 immune response in allogeneic T helper cells. The aim of this study was to investigate whether allergen-pulsed DC also induce a Th1 response in patients with an allergy of the immediate type, which might be useful for the therapy of atopic diseases. For this purpose DC were generated from peripheral blood monocytes from atopic and nonatopic individuals in the presence of GM-CSF and IL-4, and full maturation was achieved by further stimulation with IL-1β, TNF-α and PGE_2 on day 7. At the same time point DC were pulsed with grass pollen, birch pollen or house dust mite allergens and used for coculture with autologous $CD4^+$ T cells on day 9. After the second restimulation with allergen-pulsed DC $CD4^+$ T cells from atopic but not from nonatopic donors showed an enhanced IL-4, IL-5 and IL-10 production measured by ELISA, while IFN-γ production and proliferation were not different. IL-12 production and surface marker expression of DC derived from atopic and nonatopic donors did not differ and addition of anti-IL-12 antibodies during DC pulse and/or during coculture did not increase IL-4 but diminished IFN-γ production significantly. A significant shift towards a Th1 response in $CD4^+$ T cells from atopic donors could be achieved by performing the coculture on collagen type I-coated wells. This effect was due to an enhanced production of IL-12 by dendritic cells but also due to a direct increase in IFN-γ production by activated T cells. These data indicate that mature human DC induce Th1 as well as Th2 responses to allergens in atopic individuals and demonstrate that immuno-deviation away from pro-allergic Th2 responses requires additional interventions.

029

C5a is the Major Chemotaxin for Human Monocyte-Derived Dendritic Cells in Psoriatic Scale Extracts

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Dendritic cells are thought to play a crucial role in the pathogenesis of psoriasis as presenters of (auto)antigens initiating the T-cell dominated inflammatory response characteristic for this disease. It was previously shown that the number of dendritic cells in psoriatic lesions is enhanced which is due to an increased influx since these cells do not proliferate *in situ*. The aim of our study was to identify proteinaceous chemotaxins for dendritic cells in psoriatic scales using a biological/biochemical approach.

Monocyte-derived dendritic cells (MoDC) were generated from purified human monocytes by treatment with GM-CSF and IL-4 for 5 d. Extracts of pooled scale material from different donors were generated by mechanical disruption, ethanolic extraction and further purification through several HPLC-steps. Biochemical fractions were screened for chemotactic activity for MoDC using a microchemotaxis assay.

The results of our study showed that there was only one prominent chemotactic activity for MoDC present in psoriatic scale extracts which was identified as C5a by deactivation experiments and C5a-specific ELISA. C5a coeluted with MRP-14 (identified by amino acid sequencing) which was present in large amounts in scale extracts but lacked chemotactic activity.

Our data demonstrate that C5a is the only proteinaceous chemotaxin for MoDC present in psoriatic scales. Since it is known that C5a is the most potent chemotaxin for dendritic cells *in vitro* our finding seems to be of pathophysiological relevance.

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030

Analysis of Prostanoid Production by Ultraviolet (UV) Irradiated Human Blood Derived Dendritic Cells

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Prostanoids mediate the sunburn reaction and immunological changes observed after UVB irradiation (UVBR) of human skin. Epidermal keratinocytes are well known producers of prostanoids. In contrast, for dendritic cells there is only preliminary evidence for prostanoid production based on immunohistochemical detection of enzymes. We therefore assessed the functional capacity of DC to synthesize prostanoids. DC were generated from monocytic fractions of buffy coats by culture in presence of IL-4 and GM-CSF for 7 d. HPLC and ELISA analysis of supernatants of ^{3}H-arachidonic acid (AA) prelabeled and unlabeled DC identified thromboxane $(TX)B_2$ and prostaglandin $(PG)E_2$ as the major AA metabolites, while PGD_2 was produced in small amounts. Lipopolysaccharide (LPS) induced secretion of both, PGE_2 and TXB_2. UVBR alone was not able to significantly increase prostanoid release, while UVA1R selectively induced PGE_2, but not TXB_2 production. UVBR immediately prior to LPS stimulation resulted in significant increase of TXB_2 and PGE_2 release as compared to LPS alone. Time kinetics for TXB_2 and PGE_2 release paralleled each other and were maximal after 24 h. Increased prostanoid release was always associated with induction of mRNA for the cyclooxygenase II (CoxII): LPS treatment lead to moderate induction peaking at 4–8 h and downregulation after 24 h. Costimulation with UVBR resulted in superinduction, but not in extended expression of CoxII mRNA. In addition, UVA1R lead to induction of CoxII mRNA with two maxima after 2 and 12 h. In summary, to the best of our knowledge, this is the first direct demonstration of prostanoid production by human DC. UVR-induced modulation of DC prostanoid production depends on the wavelength, the type of prostanoid and on additional stimulatory conditions. Our studies indicate a previously unrecognized immunomodulatory property of DC which may be of particular relevance for T-cell activation.

031

Recombinant Gram-Positive Bacteria as Carriers of Heterologous Class 11-Restricted Antigens for Dendritic Cell Activation of T Lymphocytes

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Bacteria are actively investigated as vaccine carriers for inducing or boosting protective immune responses. In this study, recombinant *Streptococcus gordonii* expressing on the surface the C-fragment of tetanus toxin (TTFC) was tested as an antigen delivery system for human monocyte-derived dendritic cells (DCs). DCs incubated with recombinant *S. gordonii* were much more efficient than DCs pulsed with soluble TTFC at stimulating specific $CD4^+$ T cells as determimied by cell proliferation and IFN-γ release. Compared to DCs treated with soluble antigen, DCs fed with recombinant bacteria required 10^2–10^3-fold less antigen, and were at least 10^2 times more effective on a per cell basis, for activating specific T cells. In addition, DCs were more efficient than B cells at presenting soluble TTFC and remarkably more capable at presenting bacteria-associated MC. This difference was associated with a much lower capacity of B cells to endocytose soluble TTFC and phagocytose recombinant *S. gordonii*. Bacteria were also very potent inducers of DC, but not of B cell maturation, although they enhanced the capacity of DCs to activate specific $CD4^+$ T cells at concentrations that did not stimulate DC maturation. In particular, *S. gordonii* dose-dependently upregulated expression of membrane MHC, CD80, CD86, CD54, CD40 and CD83, and reduced both phagocytic and endocytic activities. Furthermore, bacteria promoted in a dose-dependent manner DC release of cytokines (IL-6, TNF-α, IL-1β, IL-12, TGF-β and IL-10) and of the chemokines IL-8, RANTES, IP-10 and Mig. Thus, recombinant Grampositive bacteria appear a powerful tool for vaccine design due to their extremely high capacity to deliver antigens into DCs, as well as induce DC maturation and secretion of T cell chemoattractants.

032

In Vitro Generation of Melanoma-Specific Cytotoxic T Cells by a Novel Subset of Circulating Dendritic Cells

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The study was undertaken to find out whether a new subset of circulating human dendritic cells (DCs) can activate blood T cells to a tumor specific CTL response.

With the help of the DC-specific monoclonal antibody M-DC8 a new population of DCs was isolated from human blood by a one step immunomagnetic procedure. These DCs were loaded with a tyrosinase-derived peptide and were cocultured with autologous peripheral blood mononuclear cells (PBMCs). After four cycles of stimulation cultured cells were tested for the presence of tyrosinase-reactive cytotoxic T cells in a chromium release assay. In addition, the recogniton of endogeneously processed tyrosinase was determined in a tumor necrosis factor release assay after contact with tyrosinase-expressing melanoma cells.

In the present study, we demonstrate that freshly isolated M-DC8+ cells efficiently presented a tyrosinase peptide and stimulated a peptide-specific cytotoxic T cell clone. Furthermore, M-DC8+ cells pulsed with the same MHC class I-presented peptide induced a melanoma-specific cytotoxic response in blood T lymphocytes from four out of six melanoma patients and from two out of four healthy blood donors. These results indicate that the newly described DCs are efficient in the *in vitro* generation of peptide-specific CTLs.

In conclusion, the easy preparation method and the high antigen-presenting capacity make these dendritic cells attractive candidates for vaccination protocols in the treatment of human cancer.

033

ATP Receptors and Ectonucleotidases on Bone Marrow Dendritic Cells (DC): Novel Pathways for Maturation and Differentiation?

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Extracellular adenosine triphosphate (ATP), which is released by exocytosis or by traumatic cell damage, affects the functions of many tissues and cell types by interaction with plasma membrane P2 purinergic receptors. On macrophages, ATP triggers inflammatory reactions as well as cell death. The effect of extracellular ATP is terminated by rapid hydrolysis by plasma membrane enzymes termed ectonucleotidases (CD73 and CD39).

In the present study we investigatedwhether P2 receptors and nucleotidases are expressed on mouse bone marrow DC, and we analysed the effects of extracellular ATP on DC maturation and function. Bone marrow cells as well as bone marrow-derived DC expressed mRNA for various P2 receptors (P2X, P2X7, P2Y, P2Y2) and mRNA for CD73 and CD39. Triggering via P2 receptors caused rapid Ca^{2+}-influx in mature and immature DC as shown by FURA-2 measurement and on single cell level by FACS. In the presence of TNF-α, ATP enhanced DC maturation *in vitro*, reflected by increased expression of MHC-I and costimulatory molecules CD80 and CD86. DC cotreated with ATP and simultanously TNF-α for 48 h had significantly stronger stimulatory capacity of allogeneic T cells *in vitro* than DC after treatment with TNF-α alone. Furthermore, ATP + TNF-α-treated DC were the most effective stimulators of allogeneic T cells *in vivo* after s.c. injection. In contrast to short-term ATP treatment long-term treatment of bone marrow cultures with ATP prevented the generation of DC under standard conditions. The rapid metabolism of extracellular ATP by nucleotidases was shown by separation of metabolites of ^{3}H-labeled ATP by thin-layer technique. Our data support that ATP acts as a novel costimulatory factor on DC maturation and function. The presence of ecto-ATPases and the findings that ATP alone has no stimulatory effect on DC suggest that ATPases protect DC from uncontrolled maturation and induction of autoimmunity.

034

Dendritic Cell (DC) Maturation by Small Fragments of Hyaluronan (HA) Involves a Highly Specific Nf-κB and TNFα-Dependent Pathway

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The glycosaminoglycan HA, build from repeating units of N-acetyl-D-glucosamine-UDP, is a major component of the cutaneous extracellular matrix. HA exists physiologically as a high molecular weight polymer (HMW-HA), but is cleaved into lower molecular weight fragments (sHA) at sites of inflammation. Here, we show sHA, generated from HMW-HA by enzymatic digestion and gel column separation to potently induce maturation of human DC generated from peripheral blood progenitors as demonstrated by comparative analysis of immunophenotype, cytokine secretion, T-cell stimulatory function, chemokine receptor expression and chemokine induced migration. We questioned, whether such DC-maturation is specifically induced by the basal component of HA N-acetyl-D-glucosamine-UDP. To address this issue a number of structurally related substances were tested for their ability to stimulate DC-maturation including UDP-fucose, UDP-galactose, N-acetyl-D-galactosamine-UDP, and chondroitinsulfate. Importantly, only N-acetyl-D-glucosamine-UDP dose-dependently induced DC-maturation, including upregulation of B7–1, B7–2, CD83 and HLA-DR, a downmodulation of CD115, and a markedly enhanced TNFα-release comparable to sHA preparations, generated from HMW-HA. Since TNFα was described to be of principal importance for DC-maturation, the TNFα-release in response to sHA-treatment was studied in detail. Kinetic analysis revealed TNFα m-RNA transcription rates to increase at 3 h and TNFα protein secretion to peak at 12 h after sHA-stimulation, respectively. Moreover, mobility shift assays (EMSA) documented NF-κB-activation 2 h after sHA stimulation, indicating that sHA-induced TNFα-release results from nuclear translocation of NF-κB. Finally, addition of a neutralizing soluble TNFα-R1 prevented all sHA-induced maturational events in DC. Taken together, these results indicate that sHA-induced maturation of human DC involves a highly specific, Nf-κB- and TNFα-dependent pathway.

035

Consequences of Cell Death: Exposure to Necrotic but Not Apoptotic Cells Induces the Maturation of Immunostimulatory Dendritic Cells

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The immune system has to contend with two types of cell death and their consequences: necrosis and apoptosis. Necrotic and apoptotic cells can be phagocytosed by dendritic cells (DCs), potent initiators of immunity. Uptake of antigen is restricted to the immature stage of DCs. Upon receipt of a maturation signal, DCs downregulate antigen acquisition and upregulate the expression of both costimulatory and MHC molecules, enabling them to activate resting T cells. Maturation can be triggered by multiple stimuli including LPS, contact allergens, bacteria and viruses, cell products and signaling molecules. We investigated whether the uptake of dead or dying cells could initiate immunity by inducing DC maturation.

Via a quantitative FACS phagocytosis assay, we first verified that immature DCs phagocytosed necrotic cells comparably to apoptotic cells. We then compared the phenotype of immature monocyte-derived DCs cultured for 7 d in GM-CSF and IL-4 with that of immature DCs cocultured with apoptotic (induction via UV-irradiation) or necrotic (induction via repeated freeze thawing) cells added at day 5. After 48 h the DCs were evaluated via FACS analysis using antibodies to a panel of antigens including maturation markers (CD83 and DC-LAMP) and molecules indicating activation (HLA-DR, CD86, CD40). We found that necrotic cells, but not apoptotic cells induced the maturation of DCs. Functional assays also confirmed this data. The stimulatory capacity of immature DCs in allogeneic MLRs and in staphyloccocal enterotoxin A specific proliferation assays was increased following coculture, primarily with necrotic and not with apoptotic cells.

Here we demonstrate that DCs are able to distinguish two types of tumor cell death, with necrosis providing a signal that will help to initiate immunity. Our data suggest new approaches to manipulate the immune system so that desired immune responses, e.g., to tumors can be induced.

036

Osteopontin (OPN) Interactions with CD44 Induce Epidermal Langerhans Cell and Dendritic Cell (DC) Migration

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Following antigen capture immature DC leave peripheral organs and migrate to regional lymph nodes to stimulate naïve T lymphocytes. We recently described that differential expression of CD 44 isoforms is critical for DC migration and DC mediated induction of contact hypersensitivity *in vivo*. OPN a ligand for CD44 is a secreted glycoprotein with multiple functions during tissue repair and immune responses, e.g., OPN is secreted by and acts as a chemoattractant for macrophages. To test whether OPN affects DC motility we performed modified Boyden chamber assays. These revealed that OPN dose-dependently induced DC migration and OPN failed to stimulate the migration of DC from $CD44^{-/-}$ mice. To confirm that OPN affects DC trafficking *in vivo* we injected OPN subcutaneously into the pinnae of mouse ears. Emigration of Langerhans cells was strongly induced by OPBN, but not by appropriate control proteins. In an *in vivo* migration assay that traces adoptively transferred DC, OPN was injected in close proximity to draining LN at the time of intradermal DC transfer. Compared to controls OPN increased significantly the number of labeled DC attracted to the regional LN. By PCR and OPN specific ELISA we found that immature migratory DC strongly expressed OPN mRNA and secreted high levels of OPN. Of note, such OPN expression was downmodulated during DC maturation, associated with a loss of motility. These findings indicate that OPN interactions with DC44 are of importance for DC trafficking to lymphatic organs.

037

P16 UV-Induced Mutations in Human Skin Epithelial Tumors

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The *INKA-ARF* locus localized at 9p21 locus encodes two alternative reading frame proteins (P16INKA and P14ARF) involved in tumor suppression via the retinoblastoma (Rb) or p53 pathways. Disruption of these pathways can occur through inactivation of *p16inka* or *p53*, or activating mutations of cyclin dependant kinase 4 gene (*Cdk4*). We searched for mutations in *p16Inka*, *p14arf*, *Cdk4* and *p53* genes in 20 squamous cell carcinomas (SCCs), one actinic keratosis (AK), and 28 basal cell carcinomas (BCCs), using PCR-SSCP. A deletion study at 9p21 locus and a methylation analysis of the *p16inka* promoter were also performed. Six different mutations (12%) were detected in exon 2 of the *INKA-ARF* locus (common to *p16inka* and *p14arf*) in five of 21 squamous lesions (24%) (one AK and four SCCs) and one of 28 BCCs (3,5%). These included four (66%) ultraviolet (UV)-type mutations (two tandems CC:GG to TT:AA transitions and two C:G to T:A transitions at dipyrimidic site) and two transversions. All of these mutations were deleterious for p16INKA, whereas only four had predictable consequence on p14ARF, but localized at nonconserved codons between human and mouse cDNA. *P53* mutations were present in 18 samples (37%), mostly of UV type. Of these, only two (one BCC and one AK) harbored simultaneously mutations of *p16inka*, but not *p14arf*. Loss of heterozygosity at 9p21 locus could be excluded in 12 of 20 SSCs (57%) and 21 of 28 BCCs (75%), including all tumors carrying a mutated *p16inka*. *P16inka* promoter methylation was excluded in 1/3 of tumors. Our data demonstrate for the first time the presence of *p16inka* UV induced mutations in sporadic nonmelanoma skin cancer, particularly in the most aggressive SCC type, and support that in these tumors, *p16inka* and *p53* inactivation are independent events.

038

Is Allelic Loss at 11q23 Required for Progression to Regional Lymphnode Metastasis in Melanoma?

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At least two different and independent tumor suppressor genes relevant to melanoma pathogenesis appear to be located on the long arm of chromosome 11 – in chromosomal bands 11q23.1-q23.2 and 11q23.3 – based on deletion mapping studies. Their loss seems to be a late event in the clinical course of sporadic malignant melanoma and may indicate a less favorable outcome. To establish the point in time of melanoma tumorigenesis where these two putative tumor suppressors become relevant we investigated allelic loss (loss of heterozygosity, LOH) in both chromosomal regions in tumors of progressing patients from which tumor samples of multiple progression steps (such as primary tumor and in-transit metastasis and regional lymphnode metastasis and distant metastasis) could be examined. We analyzed six microsatellite repeats in more than 100 tumor samples of 23 patients where at least two (10 patients) or (13 patients) tumor samples from different progression steps were available. Our findings indicate that allelic loss of 11q most frequently occurs at regional lymphnode metastasis. We found several patients that showed retained constitutional heterozygosity in in-transit metastases that occurred up to several months after regional lymphnode metastases that already displayed loss. Particularly in these patients loss of genetic material of 11q seemed to be required to enable tumor cells to spread to the regional lymph nodes. We therefore speculate that inactivation of putative tumor suppressor gene(s) located on 11q23 maybe responsible for progression of the disease to the regional lymphnodes.

039

Deletions of the p16(CDKN2) Tumor Suppressor Gene in Primary Cutaneous Anaplastic CD30$^+$ Large Cell Lymphoma

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The genetic alterations responsible for the development of cutaneous lymphoma are largely unknown. Chromosome region 9p21 contains a gene locus encoding an inhibitor of cyclin-dependent kinase 4, and heterozygous deletions of this tumor suppressor gene (p16) have been shown in a variety of malignant tumors.

We studied 11 randomly selected cutaneous anaplastic CD30$^+$ large cell lymphomas (ALCL). Several areas containing 20–50 CD30 positive lymphocytes were microdissected in each case and subjected to single step DNA extraction. Loss of heterozygosity (LOH) was performed using polymorphic markers at 9p21 (IFNA, D9S171) and 17p13 (TP53). Samples from normal cells apart from CD30 positive lymphocytes, e.g., lymphohistiocytic infiltrates or normal epidermal layer were also obtained in all cases from the same slide for comparison with the tumor samples. Expression of CD30 and T-lineage antigens (CD3, CD45Ro) was confirmed in all cases. Immunohistochemical staining for p16 and p53 was performed using the monoclonal antibody sc-1661 and DO-7, respectively.

Of the 11 informative cases, seven (64%) exhibited LOH at least for one marker at 9p21 (p16), while no allelic deletions were found for the polymorphic marker at 17p13 (p53). On immunohistochemistry loss of the p16 protein was detected in two of 11 cases. Nuclear staining for p53 protein was yielded in four of 11 cases.

Here, we provide the first evidence of the involvement of the tumor suppressor gene p16 in primary cutaneous ALCL. Whether p16 deletion in these lymphomas are associated with disease progression and if this method could serve as an early marker to detect lymphomas at an early stage needs to be adressed in future studies.

040

UVB Induction Of Vascular Endothelial Growth Factor (VEGF) is Mediated by Autocrine TGF-α Following Tyrosine Phosphorylation of Epidermal Growth Factor Receptor – Implication For Photocarcinogenesis

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Tumor progression of nonmelanoma skin cancers, the most common human malignancy, heavily depends on the formation of a sufficient tumor supporting neovasculature. Therefore we have focused our studies on the role of UVB irradiation in the regulation of vascular endothelial growth factor (VEGF), a multifunctional cytokine with mitogenic activity for endothelial cells during tumor angiogenesis. For this purpose the spontaneously immortalized HaCaT cell line which – due to mutations in the p53 gene is considered to represent an early skin cancer cell line – was exposed to the complete UVB spectrum We recently showed that maximal induction VEGF was obtained 12 h after UVB-irradiation on mRNA-level (6-fold induction) and after 24 h on protein-level (2–5-fold induction). Here, we further characterized the regulatory pathways of UVB induction of VEGF. The UVB-induction of VEGF was found to be mediated by a cluster of four SP-1 sites and an overlapping AP-2 site. In fact, partial deletion of this SP-1/AP-2 cluster resulted in the complete abrogation of promoter activity upon UVB irradiation, thus, confirming that the clustered SP-1/AP-2 sites are essential for the UVB-dependent activation of VEGF transcription. In addition, using neutralizing antibodies, we found that activation of the cluster of SP-1/AP-2 sites is exclusively conferred by the UVB inducible transforming growth factor-α (TGF-α). UVB-induction of VEGF was abrogated by incubation of HaCaT cells with an inhibitor of tyrosine kinase phosphorylation 4-[(Bromophenyl)amino]-6,7-dimethoxy-quinazoline (PD 153035) highly specific for the epidermal growth factor receptor. Since we found TGF-α to be induced 6 h postirradiation, while UVB induced phosphorylation of the EGF-R already occurred 30 min postirradiation, it is most likely that UVB induced EGF-multimerization stimulates autophosphorylation of EGF-R, thus preceding TGF-α induction and release. Using ELISA and immunohistochemistry, we showed that VEGF expression is up-regulated in normal human and mouse skin upon UVB irradiation, and also in sun exposed areas of eight squamous cell carcinomas suggesting that our *in vitro* findings may have *in vivo* relevance. Collectively, UVB irradiation results in the TGF-α mediated autocrine release of the angiogenic factor VEGF which may further sustain the growth of the tumor and promote the small size of a dormant *in situ* carcinoma to a rapidly growing malignancy.

041

Separate Importance of Transcription-Coupled DNA Repair and Global Genome DNA Repair in Short- and Long-Term Effects of UVB Exposure

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In recent years it has become clear that nucleotide excision repair is comprised of two subpathways, namely, transcription-coupled repair (TCR) and global genome repair (GGR). In order to establish the importance of TCR and GGR for UVB effects on the skin we employed hairless knockout mouse strains lacking either TCR (CS-B knockouts), lacking GGR (XP-C knockouts), or lacking both TCR and GGR (XP-A knockouts). We found that CS-B and XP-A mice, but not XP-C mice, have a 10 times increased susceptibility to sunburn. In contrast, XP-A, CS-B, and XP-C mice show the same extend of epidermal hyperplasia at an exposure level that does not induce any epidermal hyperplasia in wildtype animals. These results indicate that sunburn is triggered by persistent DNA photoproducts in transcriptionally active DNA, whereas epidermal hyperplasia can be triggered by persistent DNA photoproducts in both transcriptionally active and inactive DNA. Under chronic exposure to 80 J per m^2.day UV radiation TCR-deficient CS-B mice develop marked parakeratosis before the appearance of tumors, whereas GGR-deficient XP-C mice do not. The tumors that develop are squamous cell carcinomas (SCC) in XP-A, XP-C, CS-B, and wildtype mice, whereas in the TCR-deficient strains also a fair amount of benign papillomas is found. In XP-A and XP-C the induction of SCC is about four times faster than in wildtype littermates, whereas in CS-B the induction of SCC is about two times faster. We hypothesize that mutagenesis of (inactive) proto-oncogenes is responsible for the higher cancer susceptibility of GGR-deficient skin compared with TCR-deficient skin.

042

Genetic Modelling of Abnormal Photosensitivity in Families and in Twin Pairs

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Abnormal photosensitivity, as defined by polymorphic light eruption (PLE), is generally considered an acquired condition, despite a frequent history of PLE in one or more family members. In this study we examine the heritability of PLE using the twin model and determine its mode of inheritance in 58 pedigrees ascertained through photosensitive probands. A history of PLE was sought in 420 individuals from 58 families ascertained through PLE and actinic prurigo (AP) probands, and in 420 female twin pairs by questionnaire. The prevalence of PLE was 21% and 18% in MZ and DZ twins, respectively. A family history of PLE in first degree relatives (not including the cotwin) was present in 12% of affected *versus* 4% of unaffected twins, providing evidence of familial clustering ($p < 0.0001$). Twin data analysed using STATA software showed a kappa agreement of 0.65 ($p < 0.0001$) in MZ and 0.15 ($p < 0.005$) in DZ twin pairs, providing clear evidence of a genetic basis for PLE. Segregation analysis of family data revealed a similarly strong genetic component with PLE emerging as a multigene trait with a dominant mixed mode of inheritance. The model parameters estimate that 72% of the population carry a low penetrance PLE allele, such that only 24% of genotypically susceptible females and 13% of susceptible males have PLE. Within genotype variance is attributed in large part to a polygenic component with a heritability of 59%, and then to environmental factors. This study provides the first clear evidence of a genetic basis for PLE.

043

UVB-Repressible Genes: Mechanisms of Regulation and Characterization of New Players in the UV-Response of Human Keratinocytes

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In an ongoing systematic study we identify mediators and targets of the UVB-response in the human keratinocyte cell line HaCaT by mRNA differential display PCR (DD-PCR). Extending this analysis to normal human epidermal keratinocytes confirmed the usefulness of this approach. In addition it allows to pinpoint the differences and similarities between primary and cell line keratinocytes concerning their UV-modulated gene repertoire. As emerged from the first DD-PCR analysis we detect further members of the large group of genes which expression is down regulated in response to UVB-irradiation. Most of these genes are unknown or so far only identified as expressed sequence tags (ESTs). To analyze the repressive effect of UVB-irradiation in more detail we have completely cloned one of these genes at the cDNA and genomic level. The gene codes for a new, keratinocyte associated member of the ovalbumin family of serine proteinase inhibitors (serpins) and was termed hurpin. Hurpin shows a transient down regulation after exposure to 100 J per m^2 UVB with minimum mRNA levels 8 h after irradiation. Expression of hurpin seems to be related to the activation or proliferation state of keratinocytes, since hurpin transcripts are more abundant in HaCaT cells and in cultured primary keratinocytes, compared to the expression in normal skin. Moreover, in psoriasis, a skin disease characterized by hyperproliferation of keratinocytes and responsive to therapeutic UV-irradiation, overexpression of this gene is noted in psoriatic skin lesions compared to nonlesional skin. Northern blot analysis with irradiated and unirradiated HaCaT cells in which transcription is blocked by actinomycin D indicate that the observed UVB-mediated repression is not only mediated by transcriptional control but involves also post-transcriptional mechanisms. Future analysis of hurpin will elucidate not only further the mechanisms of UVB-repression but also the role of skin specific serpins in physiological and pathological human keratinocytes.

044

Novel Poly(ADP)-Ribose Polymerase Inhibitor with Photoprotective Activity

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UV-induced stress can trigger DNA strand breakage which activates the nuclear enzyme poly(ADP)-ribose polymerase (PARP). Rapid activation of the enzyme depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide, thus slowing the rate of glycolysis, electron transport and subsequently ATP formation. Excessive PARP activation has been shown to lead to inhibition of cellular energy-producing methabolic pathways which contributes to cell disfunction, tissue injury and necrosis.

Aim of the study was to test the photoprotective ability of nicotinic acid amidoxime derivative (BGP-15), a novel PARP inhibitor against photodamage produced in skin by single exposure to ultraviolet (UV) light.

In skin biopsies of hairless mice [CRL:hr/hr BR (n = 6)] irradiated by UV21 Philips lamps (peak at 313 nm in UVB) using a single erythematogenic (4 MED) UVB exposure the activity of self-ADP-ribosylation of PARP (determined by Western blot with anti-ADP-ribose monoclonal antibody, quantitated by Image Tool Version 1.27) proved to be excessive (signal intensity: 61 $\pm$ 6 U) compared to unexposed skin samples (2 $\pm$ 1 U). Immunohistochemistry of UVB-exposed mouse skin showed heterogenous positivities of epidermal cells with the monoclonal N/A antibody to poly ADP-ribose (Biomol Res. Lab. Inc., Plymouth). In contrast to it, pretreatment with topically administered BGP-15-containing-cream ($\geq$ 10%) proved to be protective against 4 MED UVB-induced acute skin damage. No clinical or histological signs of sunburn could be observed. The self-ADP-ribosylation of PARP decreased (21 $\pm$ 3 U, $p < 0.05$). In biopsy samples pretreated with BGP-15 only a moderate background staining could be detected by immunohistochemical examination.

The data presented suggest that the photoprotective activity of the novel nicotinic acid amidoxime derivative (BGP-15) against UVB-induced acute skinphotodamage may be associated with the downregulation of excessive activation of PARP produced by UV light.

045

Nuclear and Cell Membrane Effects Contribute Independently to the Induction of Apopotsis in Human Cells Exposed to Ultraviolet Radiation

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Ultraviolet radiation (UV) induced DNA damage is a crucial event in UV-mediated apoptosis. On the other hand, UV directly activates the apoptosis related surface molecule CD95, suggesting that UV-induced cell death is initiated also at the cell membrane. UV-mediated DNA damage can be reduced by treating cells with liposomes containing the repair enzyme photolyase followed by exposure to photoreactivating light. Addition of photolyase followed by photoreactivation after UV reduced the apoptosis rate of HeLa cells significantly, while empty liposomes had no effect. Likewise photoreactivating treatment did not affect apoptosis induced by the ligand of CD95, CD95L. UV exposure at 4°C which prevents CD95 activation also reduced the apoptosis rate but to a lesser extent. When cells were exposed to UV at 4°C and treated with photolyase plus photoreactivating light, UV-induced apoptosis was almost completely prevented. Inhibition of caspase-3, a downstream protease in the CD95 signaling pathway, blocked both CD95L and UV-induced apoptosis, while blockage of caspase-8, the most proximal caspase, inhibited CD95L-mediated apoptosis completely, but UV-induced apoptosis only partially. Although according to these data nuclear effects seem to be more effective in mediating UV-induced apoptosis than membrane events, both are necessary for the complete apoptotic response. Thus, this study shows that nuclear and membrane effects are not mutually exclusive and that both components contribute independently to a complete response to UV.

046

Antibodies to BP180 Induce the Release of IL-6 and IL-8 from Cultured Normal Human Keratinocytes

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Bullous pemphigoid (BP) is an autoimmune bullous disease associated with autoantibodies against BP180. The pathogenicity of antimurine BP180 autoantibodies was demonstrated in a passive transfer mouse model. The pathogenic relevance of antibodies to human BP180, however, has not been shown to date. Several cytokines have been suggested to be involved in blister formation of BP. We incubated normal human epidermal keratinocytes (NHEK) and GABEB cells, that lack normal BP180 expression, with IgG affinity purified from BP and healthy control sera. Levels of IL-6 and IL-8 but not IL-1α, IL-1β, TNFα, IL-10, and MCP-1 were markedly increased in the culture supernatant of NHEK compared to control IgG. This effect was dose and time-dependent and confirmed for IL-8 at the mRNA level. BP IgG did not induce the release of IL-8 (1) from BP180-deficient GABEB cells (2) from NHEK grown under differentiation promoning conditions (high Ca^{++}), and (3) when depleted of immunoreactivity with two recombinant fragments of BP180 NC16A. These data indicate that antibodies to well-defined epitopes on the human BP180 ectodomain trigger a signal transducing event that leads to expression and secretion of IL-8 from basal keratinocytes.

047

The Major Pathogenic Epitope of the Laminin α3 Subunit, in Cicatricial Pemphigoid, is Localized Within the G Domain

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Auto-antibodies to laminin-5 ($\alpha3\beta3\ \gamma2$) are mostly directed against the α3 subunit and they induce subepidermal blisters in a subset of patients with cicatricial pemphigoid (CP). We sought to determine the pathogenic epitopes of the laminin α3 subunit using GST-fusion proteins corresponding to different parts of the molecule. Five of five anti-α3 CP sera reacted with a fusion protein corresponding to G2-G3 domain of the α3 subunit. No reactivity observed with fusion proteins corresponding to other parts of the molecule. IgG reactivity of anti-α3 sera against the G domain was further confirmed by rotary shadowing images of purified laminin-5 incubated with IgG anti-α3. IgG auto-antibodies were mainly found adjacent to the globular domain of laminin-5. Moreover, antibodies directed against the G domain of α3 disrupted the dermal-epidermal junction of normal human skin as visualized by electron microscopy. This study demonstrates the presence of a common epitope on the G-domain of α3 subunit for IgG auto-antibodies in sera from patients with CP. It furthermore provides evidence for the key role of the G domain of α3 subunit in cell/matrix adhesion.

048

Healing of Patients with Bullous Pemphigoid is Associated with the Loss of Anti-BPAG2 Antibody Subclasses

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Bullous pemphigoid (BP) is an autoimmune blistering disease characterized by the production of autoantibodies directed against BPAG1 and BPAG2, two proteins of the basement membrane zone (BMZ). To investigate the apparent paradox that there is no correlation between the titers of anti-BMZ antibodies and the course of the disease, we reevaluate the immunochemical properties of anti-BMZ antibodies during the course of BP by determining their titers, antigenic specificities and isotypes in paired sera of 50 BP patients collected during the active phase of the disease (initial sera) and during remission (healed sera) after a mean follow-up period of 18.4 mo. When antiwhole IgG antibodies were used as the tracer, the mean immunofluorescence (IF) titers and the immunoblotting profile of anti-BMZ antibodies were not found to be different in initial and healed sera. However, analysis of IgG subclasses of anti-BPAG1 and anti-BPAG2 antibodies in these sera showed that whereas 78% of paired sera had the same anti-BPAG1 antibody IgG subclass pattern, anti-BPAG2 antibodies of IgG4 and less frequently IgG1 subclass, disappeared from 89% of healed patient sera. Accordingly, the mean anti-BMZ antibody titers of anti-BPAG1 antibody positive sera remained unchanged during the course of the disease, and that of anti-BPAG2 antibody positive sera decreased by seven dilutions between initial and healed sera ($p = 0.03$). Interestingly, whereas both IgG1 and IgG4 anti-BPAG2 antibodies could be detected by immunoblotting in initial sera, IF titration experiments showed that anti-BPAG2 antibodies mainly corresponded to IgG4, and their titers decreased dramatically when patients healed. These data argue for the pathogenic properties of anti-BPAG2 IgG4 antibodies in BP patients, which is in accordance with results obtained in animal models.

049

Carbohydrate Moieties Determine the Antigenicity of the Pemphigus Foliaceus Antigen

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Pemphigus foliaceus (pf) is an autoimmune bullous disease characterized by pathogenic autoantibodies (aab) directed against the extracellular domain of desmoglein I (dgI), a desmosomal cadherine type adhesion glycoprotein. Desmosomes are major cell adhesion structures of epithelial cells and their capacity to mediate cell adhesion requires the presence of sugar residues. Since dgI exhibits several N- and O-glycosylation sites, and pf-autoantibodies (aab) obviously impair the adhesive function of dgI, we asked the question, whether sugar residues might contribute to the antigenicity of the pf-autoantigen. Processing cryosections of normal human skin for immunomorphological studies we demonstrate that certain lectins Concanavalin A and wheat germ agglutinine completely inhibited the consecutive binding of pf-aab but not of a monoclonal antibody. Deglycosylation of skin sections with N-glycosidases and mannosidase but not with O-glycosidases also completely abolished pf-aab binding. Our assumption that N- but not O-glycosylation of dgI might account for this phenomenon was additionally supported by Western-blotting of epidermal protein extracts: dgI in its native form migrated to 160 kDa, as detected by both, mab-dg3.10 and pf-aab, whereas after deglycosylation dgI migrated to a molecular weight of 140–145 kDa and was only more detectable with mab-dg. Organ culture experiments displayed identical results, i.e., pf-aab binding inhibition by lectins and N-glycosidases and subsequently of blister formation. Our findings suggest that carbohydrate moieties play a decisive role in the autoantigenicity of the pf-autoantigen and indicate that the pf-autoantigen is localized on a conformational epitope of the extracellular domain of dgI.

050

Transmembrane Collagen XVII is Triple-Helical and Mediates Cell Adhesion

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Collagen XVII, a transmembrane hemidesmosomal component, is mutated in junctional epidermolysis bullosa and acts as an autoantigen in blistering skin diseases. In order to study the conformation and functions of this unusual collagen, the largest collagenous domain, Col15, of collagen XVII was produced in a recombinant expression system. Amino acid analysis of recombinant Col15 domain (rCol15) showed that over 70% of the eligible proline residues were hydroxylated to 4-hydroxyproline. The folding and thermal stability of rCol15 were probed with circular dichroism and trypsin digestions. Both assays indicated that rCol15 produced in the presence of ascorbate had a triple-helical conformation and a melting temperature of 25°C. In contrast, when synthesized without ascorbate, rCol15 showed a melting temperature of 18°C. The mutation G627V which leads to junctional epidermolysis bullosa had a striking effect on the thermal stability of rCol15, leading to partial unfolding already at 4°C. Interestingly, rCol15 promoted cell adhesion of several cell lines by a β1 integrin mediated mechanism. Epitope mapping showed that rCol15 was recognized by autoantisera of both bullous pemphigoid and linear IgA dermatosis patients.

The data demonstrate collagen XVII forms a triple-helix, but also that ascorbate is absolutely required for the correct biosynthesis and folding of this collagen. The Col15 domain can mediate cell adhesion, and its functional perturbance through genetic aberrations or autoantibody binding may cause epidermal-dermal separation in both autoimmune and inherited blistering skin.

051

A Second Type XVII Collagen mRNA Transcript in Human Keratinocytes

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The mRNA for human type XVII collagen has been reported to be a 6-kb transcript derived from the *COL17A1* gene. Here we report the presence in normal human keratinocytes of an additional *COL17A1* transcript which is approximately 0.6 kb smaller in length. Both mRNA's hybridized on Northern blot with probes directed to sequences coding for parts of the intracellular and extracellular domains of type XVII collagen. By BLAST homology search alignments, followed by cloning and sequencing, we extended the 3′ untranslated region (3'UTR) of the known type XVII collagen mRNA sequence by 877 basepairs to completion. Three BLAST identified cDNA's contained a 610-bp deletion in this new 3'UTR sequence. Northern blot analysis with a probe complementary to this deletion only identified the larger mRNA. The deletion of 610 bp in the smaller mRNA was verified by RT-PCR and sequencing. Genomic PCR showed the new sequence to be an extension of exon 56 of the *COL17A1* gene suggesting that the second mRNA is generated by differential splicing. Alignment of the 3'UTR sequences of human and mouse mRNA showed four stretches in which the degree of homology was as high as the homology between the protein coding sequences suggesting that these noncoding sequences are important for proper functioning of the messenger. In normal keratinocytes the smaller mRNA was found to be expressed at a level of 5–15% of that of the larger transcript whereas in a squamous cell carcinoma cell line this ratio was inverted, the smaller mRNA being three times more abundant than the larger mRNA.

052

Decrease in Epidermal CD44 Expression as a Potential Mechanism for Abnormal Hyaluronate Accumulation in Superficial Dermis in Lichen Sclerosus and Atrophicus

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CD44 is a polymorphic integral membrane glycoprotein which serves as the principal cell surface receptor for hyaluronate (HA), the major component of the extracellular matrix. CD44 is abundantly found in the skin and functions as a cell adhesion molecule. In a recent study we have observed a massive dermal accumulation of HA as a result of the *in vivo* selective suppression of CD44 in keratinocytes of mice using a keratin-5 (K5)-CD44 antisense transgene. Since the histological features of the dorsal skin of these transgenic mice display many similarities to those of the patients with lichen sclerosus et atrophicus (LSA) such as accumulation of a loose connective tissue in the superficial dermis, we explored the epidermal CD44 expression in the skin of 14 LSA patients by immunohistochemistry and *in situ* hybridization, and the nature of this accumulated material by alcian blue and human CD44 receptor globulin (hCD44Rg) stainings, which we compared with those of the genital skin of the transgenic mice which had not been examined in the previous study and of the skin from four lupus erythematosus, seven scleroderma and one reticular erythematous mucinosis (REM) patients. Here we show that the expression of CD44 in the epidermis of the involved LSA skin from genital and extragenital areas is significantly decreased, and in some cases completely lost, and that the accumulated substance in the superficial dermis is HA. The genital skin of these transgenic mice displays the same phenotype as their dorsal skin and skin from LSA patients, characterized by the accumulation of HA in the superficial dermis. However, keratinocyte CD44 expression was unaltered in lupus erythematosus, scleroderma and REM, despite the presence of a mucinous material in the dermis, when compared with the normal skin. These results indicate that the decrease of CD44 in the keratinocytes is the central defect in LSA constituting a novel mechanism for abnormal HA accumulation in the superficial dermis which is different from the one of other inflammatory skin conditions and mucinoses.

053

Long-Chain Unsaturated Fatty Acids Inhibit 92 kDa Gelatinase B Mediated Elastolysis. "A Novel Approach to Control Elastic Fibers Alterations During Skin Photoaging"

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Damage to skin elastic fibers is the hallmark of long-term exposure to solar ultraviolet irradiation and is most responsible for wrinkle formation. It was previously reported that induction of matrix metalloproteinases (MMPs), particularly 92 kDa gelatinase B (MMP-9), occurred at UVB doses well below those that cause skin reddening. Among MMP family, gelatinases, i.e., MMP-2 and MMP-9 were found to display elastinolytic activity, *in vitro*.

In this study we first determined the *ex vivo* elastolytic potential of both endopeptidases, using human skin tissue sections and computerized morphometric analyses, and compared it with those of neutrophil elastase.

MMP-9 was found to exhibit elastolytic activity (a maximal 50% hydrolysis of elastic fibers was attained at 50 nM concentration of enzyme) but, in such *in situ* conditions it was totally devoid of proteoglycans or collagens degrading activity. On the contrary, MMP-2 hydrolyzed proteoglycans and type III collagen but did not display elastin degrading capacity at concentration as high as 200 nM.

MMPs contain a deep (S1') hydrophobic pocket and our previous studies demonstrated that long chain unsaturated fatty acids could inhibit MMP-2 with Ki in the μmolar range. Similar investigations were performed with MMP-9. *In vitro* experiments using Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 as MMP-9 synthetic substrate showed that among fatty acids, elaidic acid (C18:1Δ9 trans) and trans-parinaric acid (C18:4Δ9, 11, 13, 15 all trans) were the most potent MMP-9 inhibitors. Similar MMP-9 inhibitory potency of those fatty acids towards elastolysis was found *ex vivo* and most importantly, in keeping with their ability to bind to elastin, they could protect elastic fibers against degradation by MMP-9.

Those fatty acids are inocuitous substances and thus could be used to prevent elastic fibers network alterations associated with photoaging.

054

Distinct Roles of Extracellular Signal-Regulated Kinase and p38 Mitogen Activated Protein Kinase in Enhancement of Collagenase-1 (MMP-1) and Stromelysin-1 (MMP-3) Expression in Dermal Fibroblasts

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We have examined the role of mitogen-activated protein kinase (MAPK) signaling pathways in the regulation of collagenase-1 (matrix metalloproteinase-1, MMP-1) and stromelysin-1 (MMP-3) expression in normal human skin fibroblasts. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are inflammatory cytokines, which induce MMP-1 and MMP-3 expression in these cells. TNF-α and IL-10 activate the extracellular signal-regulated kinase (ERK)1,2, as well as the stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK) and p38 MAPK in these cells. TNF-α and IL-1β-elicited induction of MMP-1 and MMP-3 mRNA was potently inhibited by specific p38 MAPK inhibitor SB203580, whereas blocking the ERK1,2 pathway (Raf/MEK1,2/ERK1,2) by MEK1,2 activation inhibitor PD98059 had no effect under similar circumstances. Infection of the cells with recombinant adenovirus for constitutively active MEK1 resulted in activation of ERK1,2 and JNK1, and markedly enhanced the mRNA levels and production of MMP-1 and MMP-3. Infecting cells with an adenovirus for constitutively active MKK6b (p38 kinase) specifically activated p38 MAPK and induced the expression of MMP-3, but had no effect on MMP-1 expression. Adenovirus-mediated expression of dominant negative MKK6b potently inhibited activation of p38 MAPK and upregulation of MMP-1 and MMP-3 production, and collagenolytic activity, by TNF-α and IL-1β. These results identify two distinct pairs of MAPKs capable of inducing MMP-1 and MMP-3 expression in fibroblasts: mitogen responsive ERK1,2 and JNKI, coordinately activated by MEK1, and stress-activated JNK1,2 and p38, which play an important role in controlling the proteolytic activity of normal fibroblasts.

055

Human $CD4^+$ T Lymphocytes with Remarkable Regulatory Functions on Dendritic Cells and Nickel-Specific Th1 Immune Responses

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Th1 and Tc1 lymphocytes are responsible for the development of skin allergic reactions to nickel. Here, we examined the properties of a subset of nickel-specific $CD4^+$ T cells displaying the cytokine profile (IL-10^{+++}, IL-5^{+++}, IFN-$\gamma^{+/-}$, IL-$4^{+/-}$) of T regulatory cells 1 (Tr1) and with the potential to down-modulate allergic responses to nickel. Tr1 clones were isolated from skin challenged with $NiSO_4$ and peripheral blood of nickel-allergic patients, and from the blood of healthy individuals. Tr1 clones expressed CD25, CD28, CD30, CD26 and the IL-12R β2 chain upon activation, whereas the lymphocyte activation antigen-3 was present on 50% of the clones. Monocytes precultured with Tr1 cells in the presence of nickel, or treated with Tr1-derived supernatant, exhibited a markedly diminished capacity to stimulate nickel-specific Th1 responses. Tr1 supernatants also blocked the differentiation of dendritic cells (DCs) from monocytes, as well as DC maturation and IL-12 production induced by lipopolysaccharide. As a consequence, the ability of DCs to stimulate nickel-specific Th1 and Tc1 responses was greatly impaired. These inhibitory effects were completely prevented by IL-10, but not IL-5, neutralization. Finally, Tr1 supernatants directly suppressed nickel-specific $CD4^+$, but not $CD8^+$, T cell proliferation. In aggregate, the results indicate that Tr1 cells can potently regulate the expression of Th1-mediated allergic diseases via release of IL-10.

056

Specificity Analysis of Melanoma Infiltrating Lymphocytes Used in a Clinical Trial

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Potentiel efficacy of adoptive cellular therapy by tumor infiltrating lymphocytes (TIL) depends upon the fraction of tumor specific TIL, which has been so far unknown. We assesed here how many tumor specific T cells had been infused among TIL (mean number 17 billions) to 29 melanoma patients and also which fraction of specific T cells were detected among the same TIL samples before expansion.

To this end, TIL were stimulated *in vitro* by autologous tumor cells or by TAP-deficient cells pulsed with five common HLA-A2 restricted melanoma epitopes, and the percentage of responding TIL was measured by intracellular cytokine labeling. For five patients whose TIL contained Melan-A reactive T cells, the fraction of these was also measured using fluorescent HLA-A2 antigen/Melan-A peptide tetramers.

We show that 19 out of 29 patients received a measurable number of tumor and/or Melan-A-specific TIL. Nonetheless, this number was over 300 millions for only seven patients. Interestingly, TIL from these seven patients contained before expansion the highest fractions of tumor specific TIL, mean percentage interferon-γ secreting cells: 9%, against 2.6% for others patients. Data further show that most tumor specific TIL yielded only partial response to autologous tumor cells or to the specific peptide, as shown by a defect of IL-2 secretion.

Therefore, a majority of TIL treated patients received essentially or exclusively tumor non specific T cells, this could be avoided by checking specificity and avidity of TIL before expansion.

057

Tumour Associated T Cell Epitopes Determined with a New and Rapid Technique that Combines Flow Cytometry with Mass Spectrometry

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Tumour associated T cell epitopes were identified from as little as 1 g of tumour material with 2 wk of work. MHC bound peptides eluted from tumour tissue of a patient with cutaneous T cell lymphoma were separated by HPLC and analysed for their capacity to induce cytokine production by tumour specific $CD8^+$ T cells in the peripheral blood of the patient. The reactive T cells were identified by intracellular staining for IFN-γ and flow cytometry. Active fractions were re-chromatographed by nanobore HPLC and the resulting subfractions tested for T cell epitopes as before. The peptides in these fractions were sequenced by laser-desorption/ionisation mass spectrometry. The thus identified peptides were synthesised and subjected to the IFN-γ assay. 0.59% of the peripheral blood $CD8^+$ T cells of the patient responded to the synthetic peptide confirming the identity of the natural eptope eluted from the tumour cells. This rapid procedure for T cell epitope determination will open new option for specific immunotherapy of cancer as an individual patient can benefit directly from the elucidation of the immunologic properties of his own tumour.

058

Effective Therapy of Murine A20 Tumors with A20-specific T Helper 1 Cells

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Specific anti-tumor immune reactivity mediated by CD8+ T cells has been studied extensively in various tumors. We studied the role of CD4+ T cells (Th) in anti-tumor immunity using *in vitro* generated A20-specific Th1 that produce high amounts of IFN-γ and little IL-4 upon stimulation with A20 tumor cells. At first we analyzed the efficacy of these A20-specific Th1 in tumor prevention. BALB/C mice received a lethal dose of 3×10^5 A20 cells and a single injection of 5×10^5 Th1. While 100% of the control mice died in about 35 d, 80% of the Th1 treated mice remained free of tumor. Moreover no sign of autoimmune disease was observed on autopsy after 300 d. Next we tested the A20-specific Th1 for therapy of established tumors. Five to seven days after the tumor challenge mice received one injection of Th1. At this stage of disease most other immune therapies against A20 lymphoma are inefficient. Surprisingly a single Thl injection cured up to 70% of the mice. In freshly isolated splenocytes from Th1 treated tumor bearing mice A20-specific IFN-γ production was detectable for at least 10 d after the adoptive transfer showing that the Th1 were neither deleted nor anergized by the tumor. Thus, efficient immune responses against A20 lymphoma can be generated by adoptive transfer of tumor specific Th1. This suggests that adoptively transfered Th1 provide a new promising strategy for therapy of established tumors.

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A Critical Role of TNF Receptor p55 in the Effector Phase of Contact Hypersensitivity Reactions (CHSR)

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Tumor necrosis factor alpha (TNF-α) and lymphotoxin alpha (LT-α) are known to be critically involved in the regulation of infectious, inflammatory and autoimmune diseases. However, the role of TNF-α in the induction and effector phase of CHSR is controversal. To determine the role of TNF-α in the induction and effector phase of CHSR, gene-targeted mutant mice lacking TNF receptor p55 (TNFR-1) gene were sensitized and challenged with trinitrochlorbenzene (TNCB) to induce and eliciate CHSR. Mice were sensitized with TNCB on day 0 at the abdomen and challenged on day 7 at the ear. At all times ear swelling response were up to 60% lower than in wild-type mice (C57BL/6). H&E stained sections revealed that TNFR-1 deficient (TNFR-$1^{-/-}$) mice had not only strongly reduced ear swelling, but also accumulation of neutrophils and tissue destruction. To determine whether sensitization or elicitation phase of CHSR were impaired, hapten specific T cell responses were examined. *In vitro*, $CD4^+$ T cells from immune lymph nodes (LN) proliferated identically in response to hapten modified antigen presenting cells, whether they were derived from TNFR-$1^{-/-}$ or wild-type mice. Moreover, using Enzyme-Linked-Immunospot (ELISpot) we found that the precursorfrequency of IFN-γ producing $CD4^+$ T cells was identifical in immune LN from both TNFR-$1^{-/-}$ and wild-type mice. These data strongly suggest that TNFR-$1^{-/-}$ mice had no detectable deficiency in the induction of CHSR and that TNFR-1 is critically required for efficient effector functions.

060

Frequency Analysis of Autoreactive T Helper 1 and 2 Cells in Bullous Pemphigoid and Pemphigus Vulgaris by ELISPOT Assay

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Bullous pemphigoid (BP) and pemphigus vulgaris (PV) are the two major autoimmune bullous skin diseases mediated by autoantibodies against adhesion molecules of the skin. Previous studies identified autoreactive T cells in patients with BP and PV which may be critical in providing B cell help for autoantibody production. In this study, an ELISPOT assay was established to evaluate the frequency of autoreactive TH1 and TH2 cells in patients with BP (n = 7) or PV (n = 1) and in healthy controls (n = 10). Microtiter plates were coated with anti-human-IL-5 IgG or anti-human-IFN-γ IgG prior to culturing human peripheral lymphocytes (PBMC; at 10^4–5×10^5 per well) with BP180 or desmoglein 3 (Dsg3) proteins (5–10 μg per ml) for 7 d. Cytokine producing autoreactive T cells were visualized as spot forming units. One BP patient with extensive blisters had 5.1 ± 1.5 BP180-reactive TH1 cells and 2.1 ± 1.5 TH2 cells per 10^5 PBMC. In contrast, PBMC from six BP patients in remission or under immunosuppressive therapy did not form IFN-γ- or IL-5-producing spots/ $\leq 5\times10^5$ PBMC. A patient with oral pemphigus had 4.7 ± 2.4 TH1 cells and 1 ± 0.1 TH2 cells per 10^5 PBMC reactive to Dsg3. In addition, three of 10 normals had BP180-reactive TH1 (3.3–13.8/10^5) and TH2 (1.1–1.8/10^5) cells and one normal had 9 ± 0.7 TH1 cells/10^5 PBMC but no TH2 cells reactive to Dsg3. Two of these normals carried the DQβ1*0301 allele which has been shown to restrict autoreactive T cells in PV and BP. ELISPOT reactivity correlated with [^{3}H]-thymidine incorporation in five of five patients and controls with autoreactive T cell responses. The ELISPOT assay thus seems to be promising for the quantitative and qualitative analysis of autoreactive T cell responses in BP and PV and may serve as a tool to monitor disease activity and to better understand the role that T cells play in the pathogenesis of these autoimmune diseases.

061

Expression of Killer Cell Inhibitory Receptors (KIRs) Inhibits the Cytotoxic Activity of a Cutaneous T Cell Lymphoma-Specific T Cell Clone

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T cells with KIR receptors may represent an escape mechanism that antagonizes cytotoxic T cell function. We have previously reported several tumor-specific cytotoxic T cell clones infiltrating a cutaneous T cell lymphoma (CTCL). These clones mediated a specific MHC class I-restricted cytotoxic activity toward the uncultured tumor cells and autologous long-term tumor T cell lines. In the present report, we cloned the peripheral blood lymphocytes of the same patient at an early phase, before the invasion of the blood by tumor cells. We report the rapid expansion of a new Vβ13+CD8+CD4− tumor-specific cytotoxic clone. Both the long-term T cell lines and the cytotoxic T cell clone expressed TCR-Vβ13 and Vβ13Jβ2.5. However, complete VDJ sequencing showed that the tumor lymphocytes and the nontumoral circulating clonal lymphocytes expressed different VDJ sequences. Functional studies showed that the non tumoral circulating clonal population had a strong natural killer-like cytotoxic activity on K562 target cells, whereas a weak cytotoxic activity was found on autologous tumor targets. This specific cytotoxic activity was restricted by HLA-A2, since it was inhibited by an anti-HLA A2 monoclonal antibody (mAb). In contrast, the specific cytotoxic activity of the T cell clone was strongly enhanced by the addition of an anti-HLA B/C mAb. Flow cytometric analyses showed that the T cell clone expressed high levels of several KIRs: CD158a (p58.1), CD158b (p58.2), NKB1 (p70), and CD94. Neither of these KIRs was expressed by the tumor cells and cell lines. In conclusion, we show for the first time that KIR expression may impair the efficiency of specific cytotoxic T cell responses in patients with CTCL.

062

Effector T Lymphocytes are More Potently Co-Stimulated by Type I Collagen than Fibronectin and Other Matrix Proteins

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Resting $CD4^+$ T lymphocytes purified from normal human peripheral blood (PB) by magnetic beads are potently activated by culture with coimmobilized anti-CD3 and the extracellular matrix proteins (ECMP) fibronectin (FN) and laminin (LN) but not collagen (COL) types I or III (Shimizu *et al*, *J Immunol* 145:59–67, 1990). However, most extravascular dermal T cells in inflammatory disease are likely to be functionally different from resting PB cells. We have therefore determined the reactivity to ECMP of effector T cells. Fresh, resting $CD4^+$ and $CD8^+$ T cells (98% pure) were isolated from normal PB by magnetic beads. Effector T cell lines (98% pure) were generated from the purified PB $CD4^+$ and $CD8^+$ cells by repeated rounds of stimulation with tetanus toxoid or PHA, respectively. Three-day proliferation assays with the purified resting cells in wells coated with anti-CD3 (0.1–0.5 μg per ml) and ECMP confirmed that the cells are potently activated by FN (up to 90 000 cpm), less by LN but minimally or not at all by COL I and III, confirming previous work. In contrast, and for the first time, effector $CD4^+$ and $CD8^+$ T cell lines were shown to be potently coactivated by low coating concentrations (0.6 μg per ml) of COL I (up to 120 000 cpm), whereas the same coating concentrations of FN, LN and COL III gave small responses (usually $<$ 10 000 cpm). COL I-induced effector T cell activation was dilution-related, causing major responses ($>$ 30 000 cpm) at coating concentrations of only 0.04 μg per ml, and was potently inhibited by β1-integrin antibody. Major COL I-induced responses were also seen with serum-free culture medium, indicating effects independent of serum FN. Flow cytometry showed that the T cell lines expressed all VLA integrin dimers from α1–5, including those that bind collagen. These results reveal for the first time potent, β1-integrin-mediated effector T cell activation by COL I, the major collagen of human dermis, and suggest novel approaches to immunotherapy.

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T Cell Receptors in Human Nickel Contact Dermatitis: Contact Residues for the Nickel-Induced Antigenic Determinant and the Restricting MHC Molecule

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The T cell receptor (TCR) of alpha/beta T-lymphocytes recognizes antigenic peptides embedded in MHC class I or class II molecules. Only little is known about TCR-antigen–MHC interactions for inducers of contact hypersensitivity such as nickel. The antigenic epitopes created by the metal ions and recognized by Ni-specific T cells are still not identified. In previous studies, we reported an over-representation of TCRBV17 elements in Ni-induced $CD4^+$ T cell lines from severely allergic donors. In one of these donors (IF), an additional conservation of the amino acids Arg95 and Asp96 in the CDR3B regions were observed. We have now found $VB17^+$TCR with the same CDR3B amino acid motif in a second donor (SE), confirming the important role of these sequences in Ni-allergy. By mutational analysis of these TCR residues we could show that for both donors Arg95 is required in the CDR3B sequences for Ni-specificity. Moreover, Arg95 in CDR3B could be functionally exchanged by His, indicating that the amino acid in position 95 represents a major direct contact point to Ni^{2+} ions. The finding that the HLA-restriction of one of the TCR studied was promiscuous in contrast to the others led to further investigations of TCR-MHC contacts. The pairing of the TCRA chain of the promiscuous TCR of donor SE with the TCRB chain of a nonpromiscuous TCR of donor IF could efficiently retain promiscuous Ni-recognition. This demonstrates for the first time that two TCR chains from unrelated donors can be functionally combined and, further, that promiscuous MHC restriction is mediated by the TCRA chain.

064

Papillomavirus E6 Proteins Inhibit UVB-Induced Apoptosis by Bak to Promote Skin Tumour Development

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Non-melanoma skin cancers (NMSC) which contain human papillomavirus (HPV) DNA develop primarily on body sites exposed to sunlight in EV or immunosuppressed patients, pointing to UV radiation as an important aetiological agent in the development of the disease. We have previously shown that cutaneous and EV-associated E6 proteins, from both low risk and tumour-associated HPV types, efficiently inhibited UV-induced apoptosis by both p53-dependent and independent mechanisms. The aim of this study was to determine the underlying molecular mechanisms by which diverse cutaneous HPV E6 proteins are able to inhibit apoptosis following DNA damage. An important mediator of apoptosis in epithelial cells is the Bak protein, a Bcl-2 homologue. We demonstrate, both by western blotting of extracts from cultured cells and immunohistochemistry of skin specimens, that the Bak protein is strongly induced by UVR from very low basal levels. This resulted in apoptotic cell death as measured by TUNEL in both normal and p53 null cells. In contrast, in cells expressing a cutaneous HPV E6 protein no increase in Bak protein levels was seen following UVB treatment. Mechanistic studies revealed that E6 promoted the degradation of Bak through ubiquitin-mediated proteolysis. Immunohistochemical analysis of Bak levels in NMSC biopsies showed that Bak was undetectable in HPV-positive tumours, but in contrast, HPV-negative tumours expressed Bak. Our results demonstrate that Bak induces apoptosis in cutaneous keratinocytes in response to UVB damage and that this can be abrogated by a variety of E6 proteins. Restoration of Bak function may therefore be useful in intervention against skin lesions harbouring HPV.

065

WNT-4 Expression and Function in Human Skin

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The developmental gene family WNT has been involved in various processes such as cell fate determination, adhesion molecule regulation and carcinogenesis. We have investigated which members of this family were expressed in human skin by degenerated RT-PCR, cloning and sequencing.

We found that three genes, equivalently represented in the samples analysed, were expressed in foreskin: WNT-4, WNT-5A and WNT-13. We next focused on WNT-4 function and developed fibroblasts overexpressing WNT-4 by retroviral infection. Proliferation studies showed no difference when keratinocytes were cocultured in presence of fibroblast expressing WNT-4. Using a clonogenic assay, we showed that the colony forming efficiency was not modified by WNT-4; however, qualitative modifications were observed: the percentage of holoclones was dramatically reduced, and most of them were transformed to the meroclone/paraclone type. This result was further confirmed by the analysis of differentiation marker expression: WNT-4 increased the number and the size of involucrin positive cells even in absence of calcium.

Taken together these results suggest that WNT-4 could promote the transition from the stem cell to the transit amplifying cell compartment and that WNT-4 is a strong inducer of keratinocyte differentiation.

066

WN T-1 Gene Product is Expressed by Human Epidermal Keratinocytes and Controls Their Differentiation

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Developmental genes have taken an important place in epidermal carcinogenesis over the last few years. Among them, little attention has been paid to the WNT genes which encode for secreted proteins that regulate cell fate and cell–cell interaction of multipotential cells in a variety of tissues. We have investigated the expression of WNT-1 in human adult epidermal keratinocytes either *in vitro* or *in vivo* and found that WNT-1 expression could be detected at the protein and RNA level. No expression was detected in dermal fibroblasts. We have next generated a replicationdeficient retrovirus containing WNT-1 and have infected 3T3 feeder layers. WNT-1 secreting fibroblasts used in co-culture with keratinocytes induced the expression of beta-catenin, a component of the WNT signalling pathway, within the cytosolic compartment. In addition when we used a clonogenic assay, it appeared that WNT-1 had a dual effect on the keratinocyte lineage by both supporting an enhanced proliferation rate of the stem cell population and decreasing the proliferation rate of their daughter cells, the transit amplifying cells. This latter resulted from a commitment of this cell population to the differentiation process as measured by involucrin staining.

These results provide evidence that WNT signalling is part of the network that controls keratopoiesis in adult human skin.

067

FrzB, a Natural Antagonist of the WNT Fami1y, Could Inactivate WNT-4 Signalling During Keratinocyte Proliferation

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We have recently shown that the developmental genes of the WNT family can act either as potential growth factors for keratinocyte stem cell population or as inductors of differentiation. In order to evaluate a proper role for each WNT gene, we analysed the expression and the function of their natural antagonist, FrzB.

By western blot, we showed that FrzB was expressed by keratinocytes either *in vivo* or *in vitro*. In addition, only suprabasal layers expressed this protein *in vivo*. No expression could be detected in fibroblasts. By generating a fibroblast cell line overexpressing FrzB, proliferation studies showed no difference when keratinocytes were cultured in presence or not of FrzB. Using a clonogenic assay, we showed that neither the total number of colonies was altered by FrzB nor the ratio between holoclones *versus* paraclones. However, the size of the holoclones was three times larger than in controls ($p = 0.0004$). No effect on keratinocyte differentiation, i.e., involucrin staining, was observed.

A tune balance of microenvironmental factors should be necessary to insure a correct human epithelial stem cell biology. Since we previously showed that WNT-4 activates the transition from the stem cell to the transit amplifying cell compartment, it can be proposed that FrzB can antagonize WNT-4 effects in human skin.

068

Repetin is a Novel "Fused" Protein with Calcium-Binding and Repetitive Domains Localised to Chromosome 1q21

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Epidermal differentiation is a multistage process characterized by sequential expression of structural proteins. The human repetin gene was cloned from a chromosome 1 specific cosmid library. The gene was localised to chromosome 1q21 in the region of 100 kbp between the trichohyalin and profilaggrin genes. It has three exons with a translation start site in the second exon. The protein contains 28 glutamine-rich repeats of 12 amino acids (QXXXQGQSSHYG) in the central portion. The sequence of the repeat is very similar to the one of mouse repetin but the number of repeats is considerably smaller indicating it is rather the repeat sequence which is functionally important. No repeat number polymorphism was detected in the human population. The N-terminal region shows high homology to the N-terminal domains of profilaggrin and trichohyalin and the S100 proteins. This region strongly binds calcium in overlay assays using extracts from bacteria expressing the N-terminal domain of repetin. The C-terminus is highly conserved between the human and mouse protein indicating its functional importance. Repetin mRNA is strongly expressed in methylcellulose suspension cultures but only at low levels in submerged high calcium cultures of normal human keratinocytes. Repetin is expressed in suprabasal layers of stratified epithelia but not in other epithelial tissues using immunofluorescence analysis with polyclonal antibodies directed against large N- and C-terminal fragments. Our findings show that repetin belongs to the "fused" subgroup of S100 proteins like profilaggrin and trichohyalin. The different expression patterns of repetin, profilaggrin and trichohyalin indicate that they have diverse functional roles during epidermal differentiation.

069

Interleukin-1 Affects Apoptosis of Transformed Keratinocytes Differentially

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The transcription factor NFκB recently was found to protect cells from apoptosis. Since interleukin 1 (IL-1) is a well known activator of NFκB, we postulated that IL-1 might rescue keratinocytes from apoptotic cell death. Exposure of the keratinocyte cell line KB to TRAIL (tumor necrosis factor inducing ligand) or to CD95 ligand (CD95L) resulted in apoptosis, while KB cells pretreated with IL-1 were resistant to both TRAIL- and CD95-induced cell death. Inhibition of CD95- and TRAIL-mediated apoptosis was due to activation of NFκB since KB cells transfected with a super-repressor form of the NFκB inhibitor IκB were not protected. In contrast, apoptosis induced by ultraviolet radiation (UV) was not only not prevented by IL-1 but even was significantly enhanced. The antiapoptotic effect of NFκB appears to be mediated via induction of inhibitor of apoptosis proteins (c-IAP). Whereas IL-1 reversed TRAIL-and CD95-mediated suppression of c-IAP expression, UV-mediated downregulation of c-IAPs was further enhanced. Increased apoptosis induced by IL-1 plus UV was accompanied by excessive release of tumor necrosis factor α (TNFα), indicating that enhanced cytotoxicity is due to the additive effect of these two apoptotic stimuli. Accordingly, enhanced apoptosis was reduced by an antibody blocking the TNF receptor-1. These opposite effects of IL-1 indicate that different mechanisms are involved in UV-induced apoptosis than in CD95- and TRAIL-mediated apoptosis. Furthermore, the data suggest that whether a signal acts in an antiapoptotic way or not does not only depend on the signal itself but also on the stimulus causing apoptosis.

070

Downregulated Capacitative Calcium Influx, Gap-Junctional Signaling and Enhanced ATP Mediated Calcium Entry in Cultured Psoriatic Keratinocytes

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Extracellular calcium $[Ca^{2+}]_e$ is a regulator of growth and differentiation in cultured keratinocytes. Furthermore, a Ca^{2+} gradient increasing from basal keratinocytes towards spinous layer has been demonstrated *in vivo*. This gradient is disturbed in certain dermatoses e.g., psoriasis. An increase in intracellular free calcium $[Ca^{2+}]_i$ is an early event and prerequisite for differentiation in cultured keratinocytes. On the other hand, Ca^{2+} plays a role in cell adhesion. Detached from their natural surrounding and external calcium gradient, cultured psoriatic keratinocytes were investigated by monitoring free $[Ca^{2+}]_i$, which was measured using Fura-2AM as a calcium sensitive probe and image analysis system (MCID 2). We found that psoriatic keratinocytes cultured in serum free conditions in monolayers had similar intracellular Ca^{2+} stores than healthy human keratinocytes, when all $[Ca^{2+}]_e$ was chelated with EGTA and intracellular Ca^{2+} stores from endoplasmic reticulum were mobilized with thapsigargin. Increasing $[Ca^{2+}]_e$ concentration to 1.8 mM gave a mean increase of 55 nM, SD 22.9 in $[Ca^{2+}]_i$ in psoriatic keratinocytes (107 nM, 42.3 in normal keratinocytes, $p = 0.008$), which suggests defective functioning of store operated calcium channels and capacitative calcium entry of psoriatic keratinocytes. Mechanical wounding of keratinocyte monolayer resulted in a significantly reduced Ca^{2+} -wave and rise in $[Ca^{2+}]_i$ (mean 64.1 nM, 15.1) when compared to normal keratinocytes (167 nM, 57.3, $p < 0.0005$) in low and high $[Ca^{2+}]_e$. Blocking gap-junctions with heptanol did not affect $[Ca^{2+}]_i$ rise in psoriatic keratinocytes in contrast to healthy keratinocytes, which were significantly blocked. Adding extracellular ATP resulted in a more pronounced capacitative calcium influx than thapsigargin, suggesting that IP3 mediated, P_2 purinergic signaling was enhanced. Moreover, psoriatic keratinocytes maintained their defective responses to $[Ca^{2+}]_e$ and mechanical stimulation at least to 4th-6th passage, which suggests that psoriatic keratinocytes have an inborn error of Ca^{2+} handling rather than a defect in extracellular calcium gradient, or stratum corneum barrier function.

071

The Neurotrophin Family in Human Keratinocytes

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The family of human neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT4/5. These structurally and functionally related molecules, together with their receptors, play a fundamental role in the development of the nervous system and are also involved in the homeostasis of other tissues and cell-types. Although the role of NGF as a growth and antiapoptotic factor for human keratinocytes has been extensively investigated, little is known about the other NTs. In the present study, the expression and function of BDNF, NT-3 and NT4/5 in cultured human keratinocytes were analysed. By reverse transcription-polymerase chain reaction (RT-PCR), BDNF, NT-3 and NT-4/5 mRNA were clearly detected in human keratinocytes. By the same technique the Trk-C mRNA, coding for the NT-3 high affinity receptor, was also visualized. In order to exclude possible amplification of NT or trk mRNAs derived from human melanocytes, regularly present in keratinocyte cultures, these data are now being confirmed by Northern blotting analysis. Since human keratinocytes express both NT-3 and its high affinity receptor Trk-C, NT-3 was added to culture medium and proliferation was assessed by ^{3}H-thymidine incorporation: NT-3 (100 ng per ml) significantly stimulated cell proliferation. Moreover, addition of an inhibitor of trk phosphorilation, K252, to the culture medium blocked NT-3 induced keratinocyte proliferation and also significantly reduced control keratinocyte growth. On the other hand, no effect of NT-3 on the rescue of keratinocytes from UV-induced apoptosis was observed. These results strongly suggest that a complex neurotrophin network, involving not only NGF but also NT-3, exists in human keratinocytes whereby these molecules may exert both paracrine and autocrine effects.

072

Establishment and Characterization of an Immortalized Human Sebaceous Gland Cell Line (SZ95)

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Human facial sebaceous gland cells were transfected with a PBR-322-based plasmid containing the coding region for the S-40 large T antigen. The resulting proliferating cell cultures have been passaged over 50 times to date, have been cloned, and show no signs of senescence after 4 y *in vitro*, while normal human sebocytes can only be grown for 3–6 passages. The immortalized transfected cells, termed SZ95, expressed the Simian Virus-40 large T antigen and presented an hyperdiploid-aneuploid karyotype with a modal chromosome number of 64.5. The SZ95 cell line exhibited epithelial, polymorphous characteristics with different cell sizes of up to 3.25-fold during proliferation and 6-fold at confluence, showing numerous cytoplasmic lipid droplets. The cells showed lack of or only few desmosomes and large cytoplasm profiles with abundant organelles, including vacuoles and myelin figures which indicated lipid synthesis. SZ95 cells expressed molecules typically associated with human sebocytes, like keratins 7, 13, 19, and several proteins of the polymorphous epithelial mucin family. Functional studies revealed synthesis of the sebaceous lipids squalene and wax esters as well as of triglycerides and free fatty acids, even after 25–40 passages; active lipid secretion; population doubling times of 14.5–35 h; reduced growth but maintenance of lipid synthesis under serum-free conditions; and retrieval of cell proliferation after addition of 5α-dihydrotestosterone. Retinoids significantly inhibited proliferation of certain SZ95 cell clones in the expected magnitude 13-*cis* retinoic acid > all-*trans* retinoic acid >> acitretin. Thus, SZ95 is the first immortalized human sebaceous gland cell line, whereby showing the morphologic, phenotypic and functional characteristics of normal human sebocytes.

073

Poly(ADP-Ribose) Polymerase and Proteasome Involvement in UVB-Induced DNA Repair in Normal and Transformed Human Melanocytes

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Unless repaired, DNA damage induced by genotoxic agents such as UV light lead to mutations in the genome which can result in cancer induction. Epidemiological studies have shown that solar exposure is a risk factor for melanoma. However, the underlying molecular mechanisms are far from being understood. We report here that UVB irradiation of cultured normal human melanocytes (NHM) and melanoma cells (MC) results in the induction of DNA single strand breaks (ssb) and cyclobutane pyrimidine dimers (CPD) that are eliminated by a cell process in which the DNA repair enzyme, Poly(ADP-ribose) polymerase (PARP) and the multisubunit protease complex, proteasome, are involved.

Single cell gel electrophoresis ("Comet") assay performed on NHM exposed to 40 mJ per cm^2 UVB showed that maximal level of DNA ssb occured at 60 min, which corresponds to incisions by excision-repair mechanisms. Concomittant incubation of NHM with 1 mM of either 3-aminobenzamide or 4-hydroxyquinozoline, two activity inhibitors of PARP, resulted in 50% decrease of ssb. It resulted also in impaired removal of UVB-induced CPD in NHM and MC, as determined by indirect immunofluorescence (TDM2 antibody, Dr. T. Mori, Nara, Japan) and quantitative confocal laser microscopy analyses. In a biological approach we used fibroblasts from knock-out PARP–/– mice (J De Murcia, Strasbourg, France). While PARP–/– cells were unable to repair CPD lesions on the contrary to PARP+/+ control cells, drug-inhibition of PARP activity in PARP+/+ cells resulted in blockade of CPD removal. Interestingly, UVB exposure of MHN or MC in presence of the proteasome specific inhibitors lactacystin (10 μM) or N-acetyl-leucinyl-leucinyl-norleucinal (20 μM), led to 40% inhibition of DNA ssb and maintenance of CPD levels. These results show that PARP and, for the first time, proteasome are involved in repair of UVB-induced DNA lesions in melanocytic cells. Unravelling links between these effectors may be useful for the drug control of melanocyte resistance to UV stress.

074

Analysis of Cell Signalling by MIA in Active Detachment of Melanoma Cells

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Analysing the function of MIA, a protein strongly expressed in melanoma cells but not in melanocytes, we determined strong inhibition of attachment by MIA when melanoma cells are plated onto fibronectin (Fn), laminin (Ln) and tenascin (Tn). No inhibition of attachment to collagen type I, II and IV, HSPG and vitronectin was observed. Further immunoassays showed that MIA binds to the matrix proteins Fn, Ln and Tn and thereby masks the binding sites of integrins to these ECM components. Antibodies against the active centre of the integrins a4b1 and a5b1 also crossreact with MIA, suggesting a structural homology between MIA and the active integrin binding pocket.

Many studies have shown that changes in attachment of cells do result in changes of intracellular signaling. Therefore, we analysed the NFkB, SRE, SRF, AP1 and CRE pathway for changes after addition of differing amounts of MIA protein by measuring transcription factor activity. Our results show that in melanocytes the SRE pathway is activated by addition of MIA, whereas the NFkB pathway is inhibited. In melanoma cell lines (Mel Im, B16) addition of recombinant MIA has no influence on these pathways but it activates the CRE signaling pathway. Measuring protein kinase activity MIA downregulates both, S6 kinase and csk tyrosine kinase, in melanoma cells and melanocytes. In contrast, PKC is downregulated in melanocytes, but upregulated in melanoma cells. Taken together our results show that inhibition of adhesion by MIA leads to inhibition or activation of specific intracellular signaling pathways and differs in benign and melanocytic cells.

075

Heterozygote Mutants of the Melanocortin 1 Receptor Show Reduced Tanning Whilst Homozyotes Show Red Hair

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Because variants of the melanocortin 1 receptor (*MC1R*) gene are associated with red hair, we investigated families with red hair to determine the mode of inheritance of *MC1R* associated traits. 211 individuals from 11 kindreds were studied, sequencing of *MC1R* showing that 59 of 74 (80%) with red hair were homozygous/compound heterozygous for the R151C, R160W, D294H variants or one of two insertions (ins29 and ins179), indicating that red hair is usually inherited as an autosomal recessive trait. Transfection studies in COS7 cells using lipofection confirmed the inability of alpha-melanocyte stimulating hormone to stimulate intracellular cAMP production through the R151C, R160W, D294H and R142H variants.

Importantly however, whilst red-hair is present in ~5% of the UK population, we have shown clear evidence of a heterozygote effect: presence of one mutant allele gives an odds ratio of a lower Fitzpatrick phototype category of 4.1, or decreased tanning odds ratio of 4.8 ($p < 0.01$). Odds ratios for homozygotes are > 17 and 23, respectively. Such heterozygote advantage may be the driving force for natural selection in Northern Europe at this locus, and reveals the *MC1R* to be a major determinant of skin phototype even in those without red hair.

076

Variants of the Melanocortin 1 Receptor (MC1R) are Associated with Cutaneous Malignant Melanoma Independent of Skin Type

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Melanocyte stimulating hormone (αMSH) acts via its receptor (MC1R) on melanocytes to influence melanogenesis. Variants of MC1R are associated with fair skin type and red hair and also impose an increased risk of cutaneous malignant melanoma (CMM). Since fair skin is associated with CMM the question is whether variants of MC1R are causally associated with the development of CMM or that these variants exert their effect through fair skin.

Using a case-control design, 99 patients with nonfamiliar CMM and 255 controls were included. Data on skin type were collected by questionnaire and physical examination. MC1R alleles were determined by SSCP and direct sequencing. Relative risks were estimated as odds ratios by EpiInfo and were stratified according to skin type and hair colour.

Variants of MC1R in general were strongly associated with skin type: two variants of MC1R were present in 52% of 33 patients with skin type I; in 30% of 224 patients with skin type II; in 14% of 250 patients with skin type III; and in 13% of 24 patients with skin type IV. The crude odds ratios for developing CMM in persons with one variant and two variants of MC1R, respectively, compared with no variant were 2.57 (1.26; 5.31) and 3.76 (1.71; 8.41). After analyses stratified for skin type the adjusted odds ratios (Mantel-Haenszel) were 2.47 (1.23; 5.33) and 3.07 (1.25; 7.20), respectively. The very rare variants of MC1R, Asp84Glu and His260Pro and the more common variant Arg160Trp were over-represented in patients with CMM.

Variants of the MC1R gene are likely to be causally associated with the development of cutaneous malignant melanoma, independent of skin type and red hair.

077

Ultraviolet A Radiation Rapidly Induces the Focal Adhesion Kinase Phosphorylation in a Protein Kinase C-Dependent Mechanism

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We have recently demonstrated that UVA-radiation stimulates adhesion of human dermal fibroblasts in a Protein Kinase C (PKC)-dependent mechanism. In addition, using function blocking antibodies we have demonstrated that Ultraviolet A (UVA)-induced adhesion involves integrin chains α1, α2 and α5. In the present work, we investigate whether UVA radiation activates Focal Adhesion Kinase (FAK), a non receptor tyrosine kinase associated with integrin.

Human dermal fibroblasts obtained during breast surgery were grown on plastic or collagen coated dishes and irradiated by low (20 kJ per m^2) and high (100 kJ per m^2) doses of UVA. Cells were then lysed and FAK was immunoprecipited using anti-FAK monoclonal antibody. Analysis of phosphorylation was performed by western immunoblotting, using anti-phosphotyrosine antibody and visualization was achieved by chemiluminescence. In order to investigate the role of PKC in these effects, fibroblasts were pre-incubated with the specific PKC inhibitor, GF109203X. Furthermore, by pre-incubating cells with cytochalasin D, we studied whether the cytoskeleton integrity was involved or not in the UVA effects on FAK.

We demonstrate that FAK is rapidly phosphorylated by low and high doses of UVA when fibroblasts are grown either on plastic or collagen. These effects are PKC-dependent since pre-incubation with the PKC inhibitor inhibits the UVA-induced phosphorylation of FAK. Interestingly, PKC inhibitor stimulates the phosphorylation of FAK of sham-irradiated fibroblasts grown on collagen but not on plastic, suggesting a down regulation involving PKC when fibroblasts are in contact with extracellular matrix molecules. Finally, cytochalasin D does not modify UVA-induced FAK phosphorylation when fibroblasts are grown on plastic while it does when fibroblasts are in contact with a collagen matrix. These last results demonstrate that cytoskeleton integrity is not essential for UVA-induced FAK phosphorylation.

These new effects of UVA radiation on the main signaling molecule involved in the integrin-induced pathway, i.e., FAK, could trigger changes in cellular behavior leading to early stages of photoageing.

078

Differential Expression of TIMP-2 Modulates the Invasiveness of ,elanoma Cell Lines

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Gelatinase A (MMP-2) is believed to play a critical role during progression and metastasis of various malignant tumors. The proteinase is secreted from the cell as an inactive zymogen that is activated on the cell surface by membrane-type matrix metalloproteinases (MT-MMPs). In this study we present evidence that MT1-MMP plays a key role for the *in vitro* invasiveness of malignant melanoma. Melanoma cell lines of high or low invasive potential secreted latent MMP-2 when cultured on tissue culture plastic. However, when cells were grown in floating type I collagen lattices, only high invasive melanoma cells showed activation of proMMP-2, a cellular process which could be attributed to the cell surface. This process could be inhibited by antibodies against MT1-MMP or by the addition of recombinant TIMP-2. In addition, activation of proMMP-2 was shown to be dependent on the presence of active MT1-MMP as inhibition of proMT1-MMP activation by a synthetic furin inhibitor also completely abolished proMMP-2 activation. MT1-MMP protein was detected as an inactive protein form in all cell lines cultured as monolayers, whereas in collagen gels, an additional active MT1-MMP, was detected in the membranes of high as well as nonactivating, low invasive melanoma cells. In collagen gels active MT1-MMP was detected in the membranes of both high and low, invasive melanoma cells. Cell membranes isolated from cells of high, but not of low invasive capacity, when grown in collagen gels, activated exogenous proMMP-2. However, constitutive production of TIMP-2 protein was about 10-fold higher in low invasive cells as compared to high invasive melanoma cells. In addition, upon contact to collagen, TIMP-2 production was increased in the low invasive cells and decreased in the high invasive cells, indicating that in melanoma cells TIMP-2 expression levels might regulate MT1-MMP-mediated activation of proMMP-2. As compared to low invasive cell lines, the high invasive melanoma cells displayed increased *in vitro* invasiveness of matrigel which was inhibited by TIMP-2 or by TIMP-2 containing medium conditioned by low invasive cells. These data indicate the importance of these enzymes for invasion processes and support a role for MT1-MMP as an activator of proMMP-2 in malignant melanoma.

079

Effects of Cyclosporin and ASM 981 on Platelet Aggregation

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In vitro studies were made to compare the effects of a new immunomodulatory drug, ASM 981 – an ascomycin derivative (Novartis) with cyclosporin A on ADP-induced platelet aggregation both in whole blood and in platelet-rich plasma.

Blood from 10 normal volunteers, anticoagulated with hirudin, was stirred at 37°C with either ASM 981 (final concentration 1×10^{-4} M), cyclosporin A (final concentration 1×10^{-4} M) or control and stimulated with a submaximal concentration of ADP for 2 min. Aggregation was measured as percentage fall in single platelet count using the UltraFlo-100 platelet counter. Mean aggregation of 40.5% was observed in controls. This was significantly increased ($p < 0.004$, Student's t-test) to 49.5% in the presence of cyclosporin A, but significantly reduced ($p < 0.001$) to 26.7% in the presence of ASM 981.

Aggregation and aggregate size in platelet-rich plasma were compared in a Biola aggregometer on 13 occasions in response to 1 μM ADP. Both parameters were significantly increased ($p < 0.001$) after incubation with cyclosporin A compared with controls; (aggregation, 25.1% *vs* 17.1%; aggregate size, 5.6 *vs* 3.1). When ASM 981 was compared with controls, no significant increase in either aggregation or aggregate size was seen; (aggregation, 14.3% *vs* 14.0%; aggregate size, 2.9 *vs* 2.5).

The studies show that incubation of either whole blood or platelet rich plasma with cyclosporin A leads to a potentiation of platelet aggregation *in vitro* as measured by two separate techniques. This effect seems to be platelet related since it was seen in both platelet rich plasma, where only platelets are present and in whole blood where other blood cells are present. In contrast, incubation with ASM 981 did not lead to any potentiation of platelet aggregation in platelet rich plasma. Using whole blood, ASM 981 partially inhibited platelet aggregation. The effects of these two drugs on platelet aggregation therefore appear to be quite different *in vitro*. The *in vivo* implications of these findings are unclear and the clinical relevance remains to be elucidated.

080

Ascorbic Acid and Derivatives Improve 6-Hydroxysphingenin Synthesis and Barrier Function of Human Reconstructed Skin Via a Distinct Nonantioxydant Related Activity

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Epidermis reconstructed at the air–liquid interface, whatever dermal substrate used, exhibits morphological and biochemical features very close to those observed *in vivo*. However, its lipid composition and structure are different, and consequently, the epidermal barrier properties are impaired. It has recently been shown that ascorbic acid improves lipids synthesis as well as their organization within the *stratum corneum* in reconstructed skin. The objective of this study was to characterize the activity of vitamin C and its derivatives on epidermal lipogenesis and barrier function, using EPISKIN as an *in vitro* skin model. The reconstructed epidermis supplied after 6 d of culture was grown for additional 6 d at the air–liquid interface in the presence of different vitamin C derivatives or antioxydant molecules (10^{-4} M). Compounds were either added to the culture medium, or applied topically. The incorporation of [^{14}C]-acetate exhibited an increase of total glucosylceramides and ceramides fractions synthesis. Epidermal lipid quantification after HPTLC showed specific dose dependent increase of hydroxylated ceramides fractions. Vitamin C and its derivatives enhanced ceramides linked to α-hydroxylated-fatty acid, i.e., ceramides 5, 6 and 7, as well as fractions containing hydroxylated sphingoid base, i.e., ceramide 4, ceramides 5,5 and 7. HPLC analysis of sphingoid bases involved in sphingolipids, using fluorescence detection, demonstrated the presence of greater amount of C18 and C20 6-hydroxysphingenin in vitamin C treated samples. Most of vitamin C derivatives were able to increase lipid synthesis. The activity of ascorbic acid could not be attributed to an antioxidant mechanism as revealed by the use of various antioxydants. Finally, penetration studies revealed that the improved sphingolipids synthesis, following ascorbate derivatives treatment, directly enhanced the barrier properties of reconstructed human skin.

081

Do Allogenic Fibroblasts Survive Transplant when Placed in a Dermal Equivalent for Acute Wound Healing?

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The pursuit of an "off-the-shelf" skin replacement for the treatment of burns, giant naevi excision and other large area defects continues. Commercially, dermal replacements preseeded with allogenic fibroblasts are available. However the fate and function of these cells remains unclear. The aim of this study was to investigate whether allogenic fibroblasts survive transplantation and their contribution to wound repair.

The study was carried out on acute wounds, using an immunocompetent porcine model, with chambers. Dermal equivalents composed of an esterified hyaluronan scaffold, either acellular or containing allogenic male fibroblasts, were transplants on full thickness wounds. One week, after application, the wound were covered with either autologus split thickness skin grafts or laserskin (an autologus keratinocyte delivery system) dividing the study into four groups ($n = 6$). Wounds were examined, photographed and biopsied at weekly intervals. A male specific Y probe was developed to identify allogenic cells by PCR. Immumohistochemistry was performed to analyse the resulting dermis and epidermis.

Clinical analysis showed that the presence of fibroblasts did not improve clinical "take rates" of epithelium. Split thickness skin grafts produced significantly better "take rates" than cultured keratinocyte grafts. Allogenic DNA was not detected in the wounds 7 d post grafting, as determined by PCR. Immunohistochemistry revealed no differences in the inflammatory response between the four groups.

We conclude allogenic fibroblasts do not survive transplantation. The presence or absence of preseeding the dermal graft had no influence on both the clinical "take rate" and the quality of the resulting dermis and epidermis. Therefore, the use of preseeded dermal substitutes in the treatment of acute wounds is questionable.

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Not submitted

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The Skin Phenotypes of Dominant Connexin 26 Mutations

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Epidermal connexin 26 (Cx26) is associated with hyperplasia. Recessive mutations in Cx26 which cause prelingual deafness in homozygotes (DNFB1) appear not to affect the skin, but dominant mutations have recently been reported in deafness associated with palmoplantar keratoderma (PPK). We have studied two such pedigrees: in one, an in-frame deletion causes loss of a glutamate (delta 42E) and severe deafness with diffuse PPK; in the other, a missense mutation causes an amino acid substitution (D66H) and milder deafness with mutilating PPK (Vohwinkel's syndrome). Confocal microscopy of Cx26 in skin biopsies from an affected individual from each family revealed staining at points of cell-cell contact in basal and suprabasal layers of involved palmar/plantar epidermis. In one case (D66H) cytoplasmic deposits of Cx26 were present in the granular layer. In contrast to controls, Cx26 was seen in the basal layer of uninvolved forearm epidermis. Cx43 distribution was normal. Thus the mutations did not prevent Cx26 or Cx43 assembly into membrane plaques, but resulted in abnormal accumulation of Cx26. To study effects of Cx26 mutants on gap junctional intercellular communication, keratinocytes were cultured from clinically normal forearm skin of each affected individual, and cell-cell coupling was analysed by microinjection of Lucifer Yellow-CH. Passage-2 and −3 cells showed a low level of coupling in 0.1 mM Ca medium; this increased 3-fold after 24 h in 1.5 mM Ca medium, as in normal keratinocytes. Mutant Cx26 may act only in specific conditions or perhaps subtle changes in function were not detected. Further studies should establish whether the Cx26 mutants interact with wild-type Cx26 or with other epidermal connexins.

084

Mild Junctional Epidermolysis Bullosa in Dogs. A Natural Model for Model for Somatic Gene Therapy

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In humans inherited junctional epidermolysis bullosa (JEB) encompasses a group of mechanobullous diseases that manifest with blistering and erosions due to a defective in hemidesmosomes (HD) composants or their principal ligand laminin-5, leading to cleavage in the lamina lucida of the epidermal basement membrane and often to death of patients (JEB the type Herlitz). Most cases of non lethal JEB (mild JEB) have been related to a deficiency of either laminin-5, collagene XVII (BPAG2, BP180) or integrin β4. At present there is no effective treatment for these deseases that may be associated with a normal life span but produce blisters wound that are chronically difficult to head.

We have recently identified a breed of German shorthaired pointers with littermates presenting with a mild form of JEB. These dogs present a generalized skin fragility resulting in acral, auricular and oral erosions and ulcers. Ultrastructural examination of noninvolved skin reveals the presence of well-formed HD; in blistered skin the dermo-epidermal separation occurs within the lamina lucida with absence of cytolysis. Immunohistological analysis of nonlesional skin and cultured keratinocytes from three JEB dogs detected a reduced expression and secretion of the laminin-5 α3 and γ2 chains respect to the normal reactivity of the other known antigens of the basement membrane zone. Western blot and immunoprecipitation analysis of the medium conditioned by the JEB keratinocytes also reveled a marked reduction of laminin-5 secretion respect to an healthy control. These results therefore suggest that laminin-5 γ2 or α3 chains are the candidate genes in this case of mild JEB. Sequence analysis of the canine wild-type g2 chain has been established and showed 85% identities between canine and human at the nucleotide level. The screening for genetic mutations in the γ2 cDNA of JEB dogs showed no differences in respect to the wild-type counterpart. Retroviral vectors expressing the human laminin-5 γ2 or β3 chains do not complement the adhesion defects of these canine JEB keratinocytes. However immunofluorescence, western blot and immunoprecipitation analysis of these modified JEB keratinocytes showed expression, association and secretion of the recombinant polypeptide with the endogenous chains. An amphotrophic retroviral vector expressing the laminin-5 α3 chain is now under construction in order to test a phenotipic reversion of the JEB keratinocytes.

These animals are of interest because they constitute a potential natural model system providing with the opportunity of performing autologous graftings of epithelial sheets reconstructed with JEB keratinocytes reverted *in vitro*.

085

Transduction of Human Keratinocytes with a Lentiviral Vector

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Persistent gene expression has frequently been a problem in gene therapy due in part to inadequate introduction of genes into slow dividing stem cells. Unlike traditional retroviral vectors that target dividing cells, the new lentiviral vectors are able to deliver genes into nondividing cells. While retroviruses require mitosis for dissolution of the nuclear membrane to achieve genomic integration, lentiviral vectors have at least two accessory genes, MA and vpr, that enable genomic integration by interacting with the nuclear import machinery and mediating the active transport of the viral preintegration complex through the nucleopores. The goal of this study is to determine if lentiviral vectors can transduce keratinocytes (KC) and achieve sustained gene expression. In order to produce replication-defective lentiviral vectors, we transiently cotransfected three separate plasmids into 293T human kidney cells: a packaging plasmid (pCMV8.2 or pCMV8.9) containing all structural and transactivating viral factors (except envelope); an envelope plasmid providing either an amphotropic (from murine leukemia virus) or the vesicular stomatitis virus G-glycoprotein (VSV-G) envelope; and a transfer vector (pHR'GFP) containing a green fluorescent protein indicator gene, which is packaged into the lentiviral vector, and ultimately integrates into the target cell genome. At different time points following plasmid transfection, viral titer in the supernatant was analyzed by ELISA assays of p24 Gag antigen (100–270 ng per ml) and by transduction efficiency of 293T cells. Viral supernatant was taken at 48–72 h after plasmid transfection and used to transduce KC. Initial studies demonstrate that primary human KC can be transduced by lentiviral vectors, pseudotyped with VSV-G envelope, with a transduction efficiency over 95% by flow cytometry analysis. After optimization of KC transduction, lentiviral vectors will be compared to traditional retroviral vectors to asses which are best at achieving long-term expression.

086

Ectopic Expression of Naked Plasmid DNA – an Important Safety Aspect for Skin Gene Therapy

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Skin gene therapy is increasingly being investigated for therapeutic and vaccination purposes. However, it is still unknown, if the injected naked DNA is disseminated and expressed at distant sites. Therefore, we examined the presence and expression of such DNA following intradermal injection of a high dose (1 mg) of naked CMVβ-Gal plasmid DNA into the hind leg of four 15 kg pigs in various organs and at different time points (day 1, day 3 and day 11). We showed by PCR and Southern blot analysing genomic DNA that the injected plasmid DNA was transported to several distant organs and remained there for at least 3 d. Specifically, muscle (four of four samples), draining lymph node (two of two), brain (three of three), ovary (four of five), kidney (three of three), spinal cord (one of two), thyroid (four of four), stomach (four of five), small intestine (three of four), large intestine (two of two), bone marrow (one of four), spleen (four of seven), uterus (three of four), diaphragm (two of two) and the injection site (four of four) contained plasmid DNA as detected by PCR up to 3 d postinjection. On day 11 plasmid DNA was only detected at the injection site, in skin 3 cm away (two of four), in skin 12 cm away (two of four), uterus (five of eight), muscle (three of six), and diaphragm (four of six). Genomic integration events have not been detected in any of the analysed tissues by Southern blotting.
Expression was detected at the injection site, in skin 3 cm away, in skin 12 cm away up to day 11 by RT-PCR. In addition, on day 3 ectopic expression was found in draining lymph nodes and muscle. In conclusion, these results demonstrate the widespread dissemination upon intradermal injection of a "superdose" of plasmid DNA in a relevant model for skin gene therapy and show the lack of ectopic expression at later time points providing important data on the safety of naked DNA injections.

087

The Transgene Determines the Nature of the Infiltrate after Canary Pox Virus Mediated IL-2 and GM-CSF cDNA Transfer into Melanoma Metastases

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Canary pox virus (ALVAC) has been used for vaccination for years. A recombinant ALVAC encoding the genes for human IL-2 or GM-CSF was used for the intralesional treatment of melanoma metastases.
Nine patients with cutaneous melanoma metastases were included and treated with repeated injections of ALVAC-IL-2 or -GM-CSF. We monitored the local and distant changes induced by ALVAC/IL-2/GM-CSF using histology and immunohistochemistry (melanoma markers: S-100, HMB-45, MAGE-3; T cell markers: CD3, CD8, CD4, CD43, CD45R0; macrophages markers: MAC368, CD68; antigen-presenting cells: S100, CD1A). To document systemic inflammatory reactions we used serological immunoparameters including b2 microglobulin, neopterin, CRP, and the serum levels of IL-2 and GM-CSF.
Repeated ALVAC-IL-2 was well tolerated (side-effects: fever, local reddening and swelling) and resulted in local regressions in ALVAC-IL-2 injected sites. The pronounced mononuclear infiltrates consisted of T-cells (CD4+ and CD8+) and macrophages. There was an increase of CRP, IL-2, neopterin and soluble IL-2 receptor serum levels. In two out of four patients, IL-2 was found in the serum 2–4 d after ALVAC-IL-2 injections, but not thereafter, or before therapy. ALVAC-GM-CSF did not induce tumor regressions. The infiltrate contained more eosinophils, neutrophils, macrophages and CD4+ cells, but less CD8+ lymphocytes compared to ALVAC-IL-2 treated lesions. GM-CSF was not detectable in the serum.
We conclude that ALVAC-IL-2 therapy can induce local tumor regressions at injected sites. The comparison of the local infiltrates after ALVAC-IL-2 or ALVAC-GM-CSF clearly demonstrates that the transgene is crucial for its composition.

088

Human *LAMA3A* Regulatory Region Reveals Cell Type-Specificity Through AP-1 Binding Sites

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Laminin-5, an adhesion ligand of specialized epithelia presents a complex transcriptional regulation under both physiological and pathological conditions. We have cloned the 5'-flanking region of the gene coding for the human α3 A chain (*LAMA3A*) and analyzed its promoter activity. Transient expression of a luciferase reporter gene under the control of serially deleted 5'-flanking sequences revealed that the proximal promoter which contains three AP-1 sites was sufficient for keratinocyte cell type-specific expression. A single copy of this 204 bp-genomic fragment was able to confer high keratinocyte-specific expression to TK heterologous promoter. Simultaneous mutations of the three AP-1 sites cooperatively abated promoter activity and keratinocyte-specific stimulation of the TK heterologous promoter. Removal of the sequences located between the AP-1 sites does not alter significantly the cell-specific activity of the promoter, although the distance between the AP-1 sites seems critical for optimal transactivation in keratinocytes. These results provide evidences that the AP-1 sites in the promoter play a crucial and indispensable role in keratinocyte-specific expression of the gene. EMSAs showed that each AP-1 site formed a single protein-DNA complex containing Jun/fos in keratinocytes and in fibroblasts with identical binding affinities. Fos and Jun protein levels are identical in the two cell types and do not show any difference in overall phosphorylation states. We have further shown that oxidation-reduction modification of AP-1 could not explain the difference in cell-specific transactivation and that methylation state of genomic DNA cannot be responsible for that cell specificity. Therefore, these results suggest the presence of an epithelial-type specific nuclear protein which might interact with the AP-1 complexes only when all 3-dimensional requirements involving contacts wit the DNA and the AP-1 proteins are satisfied. The identification of such an epithelial-specific cofactor should further our understanding of the molecular mechanisms regulating *LAMA3A* gene and more generally epithelial gene expression.

089

Dysfunction of AP-1 Transcription Factor is Responsible for Increased GM-CSF Gene Expression in Keratinocytes of Atopic Dermatitis Patients

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Previous studies have demonstrated that keratinocytes (KC) cultured from atopic dermatitis (AD) patients release exaggerated amounts of GM-CSF in response to various stimuli. In this study, we investigated the molecular mechanisms involved in this abnormal function. In contrast to KC cultured from nonatopic controls, GM-CSF mRNA was detectable in unstimulated cultures of AD KC, and PMA induced an earlier and much stronger GM-CSF mRNA expression in AD KC, although GM-CSF mRNA degradation kinetics was similar in both KC types. Using reporter gene (CAT) analysis, the critical region of GM-CSF promoter, spanning 91 nt upstream of the cap site, was shown to confer constitutive as well as PMA-induced regulation of transcriptional activity in KC. Following PMA stimulation, CAT activity increased 10/15-fold, and AD KC exhibited significantly (2/3-fold) higher levels of CAT activity than control KC. Both NF-κB and AP-1 consensus regions of GM-CSF promoter were essential for high level transcriptional activity, since mutation of either site reduced CAT activity by > 90%. EMSA experiments showed that nuclear proteins from unstimulated KC of both atopic and control subjects formed very low amounts of specific NF-κB and AP-1 complexes, and that PMA determined a prominent increase in the levels of both complexes. However, AP-1, but not NF-κB induction was stronger and reached its peak intensity earlier in AD KC. Western blot analyses revealed that all Fos and Jun proteins were constitutively present in KC, and PMA stimulated AD KC to an earlier and stronger appearance of phosphorylated forms of c-Fos and of Jun B triplet. A more pronounced activation of AP-1 can fully explain abnormal expression of GM-CSF and other cytokines in AD KC, and suggests the existence of a molecular mechanism implicated in the specific targeting of atopic inflammation to the skin.

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Molecular Genetic Evidence for Evolutionary Selection of the Red Hair (MC1R) Phenotype

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Pigmentation is one of the most polymorphic human traits and genetic studies of this aspect of cutaneous biology may provide powerful insights into human migration and natural selection. The human MC1R gene is a major determinant of phototype, with three alleles conferring a relative risk for red hair of 6 when present as a heterozygote and > 30 when homozygous or compound heterozygous. To understand the evolutionary significance of phototype we have conducted extensive genetic epidemiological studies. We have sequenced the MC1R coding region from 300 chromosomes of individuals from Papua New Guinea, The Ivory Coast, Japan, India, Sweden and Ireland; also Eskimos from Canada and Negros from America, Africa and The Gambia.
We have identified 15 segregating sites, five of which are synonymous and 10 nonsynonymous. A striking degree of polymorphism is present only in the Northern European populations, with the ratio of nonsynonymous:synonymous sites (9:2) providing strong evidence of selection. Comparison of human and chimp DNA revealed six synonymous and eight nonsynonymous changes suggesting that constraint may be operating in the African population. These results suggest that the MC1R gene will provide a fertile ground for future population genetic studies and studies of human migration.

091

Detection and Quantification of Antigen-Specific T Lymphocytes in Peptide Treated Melanoma Patients by Flow Cytometry Based Detection of Intracellular Cytokine Expression

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The identification and characterization of antigen-specific T lymphocytes is central for the analysis of the intended immune response in various immunotherapies of cancer. Earlier methods such as limiting dilution analysis or ELISPOT are time consuming, insensitive and labor intensive, but most importantly they do not detect the antigen specific T cells directly. We have established a technique for the detection of antigen specific T cells in peripheral blood which is based on intracellular IFNγ expression. This technique has the unique advantage of providing a rapid simultaneous determination of cytokine production of defined leucocyte subsets in peripheral blood. We have applied this system here for the first time in tumor patients. Four patients with metastatic melanoma which were treated with tumor specific peptides were chosen for this study. The treatment consisted of intracutaneous injections of HLA-A1 (MAGE-1, MAGE-3) or -A2 restricted peptides (MAGE-3, gp100, Melan A) mixed with appropriate T helper antigens. Before treatment, after 24 and 48 h individual peptides (10 μg per ml) for the activation of peptide specific T cells or control peptide were added to heparinised whole blood samples and incubated for 6 h in the presence of the secretion inhibitor Brefeldin A (10 μg per ml). Cells were than permeabilized and stained with IFNγ specific antibody as well as with antibodies to T cell markers and analyzed by flow cytometry. In all patients peptide specific cells could be detected. In the HLA-A1+ patients the ranges were 0.23% to 1.73% for MAGE-1 and 0.12% to 1.57% for MAGE-3. In the HLA-A2+ patients the ranges were 0.12% to 2.83% for MAGE-3, 0.08% to 0.57% for Melan A and 0.03% to 4.69% for gp100. A several fold increase of specific T cells was seen in 3/4 patients after vaccination. The phenotype of IFNγ producing cells was determined in a three colour analysis as CD8+, CD69+ T lymphocytes. In conclusion, we were able to detect directly tumor peptide specific CTLs in the peripheral blood of melanoma patients.

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Inhibition of Cutaneous Lymphocyte-Associated Antigen (CLA) Expression by Diversion of *O*-Glycosylation Using Glycoside Based Primers

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Cutaneous lymphocyte-associated antigen (CLA), the most relevant adhesion molecule for the recruitment of T-cells into the skin, has recently been suggested to be produced by post-translational glycosylation of P-selectin glycoprotein 1 (PSGL-1) by the action of the enzyme α1,3-fucosyltransferase VII (FucTVII). α1,3-Fucosyltransferases catalyze the formation of sialyl Lewis^X (sLe^x) from sialyl *N*-acetyllactosamin. Glycoside-based primers represent a class of potential inhibitors of sLe^x synthesis on *O*-linked glycoproteins. They resemble biosynthetic intermediates and act as wrong substrates thereby diverting the synthesis of oligosaccharide chains. We have sought to assess if the glycoside based primer acetylated GlcNAcβ1-3Galβ-*O*-naphthalenemethanol (GlcNAcβ1-3Gal-NM) could divert the oligosaccharide synthesis leading to CLA expression in superantigen activated T-cells. As determined by flow cytometry in TSST-1 (100 ng per ml) stimulated PBMCs GlcNAcβ1-3Gal-NM inhibited the expression of CLA on CD3$^+$ cells in a dose dependent manner at nontoxic concentrations ranging from 1 to 100 μM (n = 7) with a maximum effect at 100 μM (8,9 ± 4,5 *vs* 29,8 ± 4,8); these levels were even below the expression of CLA found in resting T-cells (10,2 ± 2,6). A control glycoside based primer (Galβ1-3GalNAcα-NM) had no inhibitory effects. The expression of the protein backbone PSGL-1 remained unaffected by GlcNAcβ1-3Gal-NM treatment, as well as the expression of ICAM-1, a glycation independent TSST-1 inducible protein. Through the inhibition of oligosaccharide synthesis an accumulation of *O*-linked αGalNAc is expected to occur at the cell surface. A more than fourfold accumulation was indeed found on GlcNAcβ1-3Gal-NM treated TSST-1 activated T-cells as determined with FITC-labeled *Helix pomatia* lectin. We have previously shown that CLA expression is inhibited by antioxidants through inhibition of FucTVII. Here we present a second distinct approach to inhibit CLA expression. The combined inhibition of CLA at different sites could lead to novel therapeutic tools to more specifically target skin homing T-cells.

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CTACK, A Skin-Specific Chemokine that Preferentially Attracts Skin-Homing T Cells

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In contrast to naïve lymphocytes, memory/effector lymphocytes can access nonlymphoid effector sites and display restricted, often tissue-selective migration behavior. The cutaneous lymphocyte-associated antigen (CLA) defines a well described subset of circulating memory T cells that selectively localize in cutaneous sites mediated in part by the interaction of CLA with its vascular ligand E-selectin. E-selectin is broadly expressed in inflamed endothelium; thus, specific infiltration of skin by CLA$^+$ T cells must require additional cues. Here, we report the identification and characterization of a new C-C chemokine, cutaneous-T-cell-attracting chemokine or CTACK. Both human and mouse CTACK are detected only in skin. Specifically, CTACK is found in the mouse epidermis and in human keratinocytes. Finally, CTACK selectively attracts CLA$^+$ memory T cells and is expressed in psoriasis and atopic dermatitis. Taken together, these results suggest an important role for CTACK in recruitment of CLA$^+$ T cells to cutaneous sites. CTACK is the first chemokine described that is predominantly expressed in the skin and that selectively attracts a tissue-specific subpopulation of memory lymphocytes.

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Isolation of a Fungal Pathogen Pattern Associated Molecule

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In previous studies we identified a proinflammatory leukocyte-activating lipid termed "LILA", which is present in all fungi and yeast preparations investigated so far, but not in mammalian cells. In order to determine whether LILA exhibits apart from its leukocyte-activating properties also keratinocyte stimulatory properties HaCat-keratinocytes were treated with LILA. The subsequent induction of mRNA encoding several cytokines and chemokines by LILA-stimulation was investigated by RT-PCR. As a result, LILA was found to induce mRNA for IL-1α, IL-1β, the chemokines IL-8, IP-10, MCP-1 and RANTES but not TNF-α or the chemokines MCP-3 and ENA-78. Furthermore mRNA for the IL-8 receptor CXCR-2 but not CXCR-1 was upregulated. Purification of LILA preparations and ^{1}H-NMR as well as mass spectrometrical analysis revealed 1-oleoyl-lysophosphatidylcholine (LPC) and 1-palmitoleyl-LPC as major components. A synthetic 1-oleoyl-LPC did not show any leukocyte and keratinocyte-activating properties indicating that an as yet unidentified minor component is responsible for LILA-activity, which is currently being further purified.

We conclude from our findings that a fungus-specific lipid (LILA) is capable to induce both direct leukocyte activation as well as induction of proinflammatory cytokines in keratinocytes. This occurs via as yet unidentified molecular Pattern Recognition Receptors (PRRs) on leukocytes and keratinocytes and thus represents a novel Pathogen Pattern Associated Molecule (PAM) of fungi.

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The C-Terminal Tripeptide LAR of C3a Anaphylatoxin is Crucial for the Activation of Human Eosinophils

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As a proinflammatory mediator C3a evokes a variety of *in vivo* and *in vitro* effects via a specific G_i protein coupled receptor which shows a broad expression in different tissues and also cells of the peripheral blood like eosinophils. These observations support an important role for C3a in diseases typically associated with a peripheral blood or tissue eosinophilia such as allergic asthma and atopic dermatitis. Synthetic human C3a analog peptides with variations at the C-terminal effector domain have been evaluated with respect to their binding affinity and signaling potency on human eosinophils. Flow cytometric analysis and RT-PCR revealed that the C3a receptor is constitutively expressed on human eosinophils. Peptides bearing an N-terminal Fmoc-Ahx motif were the most powerful peptides tested. Amino acid replacements in the conserved C-terminal pentapeptide decreased binding affinity and functional potency substantially. In addition, synthetic C3a analog peptides induced C3aR internalization, led to transient changes of $[Ca^{2+}]_i$ and did release reactive oxygen species in human eosinophils indicating the *in vivo* relevance of C3a related sequences. The tripeptide LAR was found to be essential for C3a receptor binding on human eosinophils. Moreover, the putative binding motif of C3a anaphylatoxin is also crucial for the induction of biologic effects in the human system such as changes of $[Ca^{2+}]_i$ and the release of reactive oxygen species. This study demonstrates that the carboxyterminus is important for the interaction with the C3aR and the biologic potency of C3a anaphylatoxin in the human system and plays a key role in the activation process of human eosinophils.

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Ezematous Skin Lesions of Allergic Contact Dermatitis and Atopic Dermatitis Express a Similar Biphasic Cytokine Pattern

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Allergic contact dermatitis (ACD) has been associated with a Th1 and atopic dermatitis (AD) with a Th2 type cytokine profile. Recent studies however, suggest that Th1 type cytokines such as IFN play an important role in the pathogenesis of chronic AD skin lesions. In this study we show that Th2 type cytokines such as IL-4 are also expressed in acute ACD patch test lesions. We examined the mRNA expression of IL-4, IFN, CD3, CD4 and β-actin of skin biopsies taken from sites of inflammatory skin lesions. In patch test lesions IL-4 was markedly expressed in 87% whereas a strong expression of IFN could only be found in 56%. In chronic ACD IFN expression clearly exceeded IL-4 expression. In all chronic AD biopsies IFN was detected, but no or very weak expression of IL-4. In addition to the mRNA analysis, intracellular cytokine contents of freshly isolated skin derived lymphocytes from nonatopic patients with ACD were determined by flow cytometry. An IL-4 producing subpopulation of 7,2 ± 2,15% was found in permeabilized skin infiltrating lymphocytes from patch test lesions which had not further been stimulated *in vitro*. In contrast, blood lymphocytes did not bind anti-IL-4 antibodies without prior *in vitro* stimulation. The majority of nickel-allergen specific T cell clones (TCC) derived from acute patch test lesions secreted IL-4 which was in contrast to TCC from chronic lesions or peripheral blood. Our finding point to a local IL-4 expression in skin-infiltrating lymphocytes in acute ACD/patch test lesions which is not associated with atopy or enhanced IgE production. This further supports the view that there is no fundamental difference in the cytokine pattern expressed *in vivo* at sites of acute vs. chronic inflammation between AD and ACD.

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Identification of Human Neutrophil α-Defensin 1 (HNP-1) as Major Antimicrobial Peptide of Healthy Skin Keratinocytes

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We recently discovered human β-defensin 2 as inducible antimicrobial peptide that is produced by keratinocytes only upon stimulation with proinflammatory compounds. In order to identify antimicrobial peptides produced by healthy skin we analyzed extracts obtained from epidermis, heel callus as well as supernatants of growing primary keratinocytes for the presence of antimicrobial peptides using a plate diffusion assay system. As a result we found that epidermis extracts contained antimicrobial activity, that bound to a heparin-sepharose-column indicating the presence of cationic antimicrobial peptides. Preparative reversed phase HPLC separation revealed a number of activity peaks. One major peak was purified to homogeneity by micro-HPLC. N-terminal amino acid sequence analyses revealed as major sequence AXYXRIPAXI and as minor sequence XYXRIPAXI, which is identical with sequences published for human neutrophil α-defensins (HNP) 1 and 2, respectively. Because it has been a dogma that HNP-1 and 2 are solely produced by neutrophils, we investigated also normal heel callus extracts as well as supernatants of cultured primary keratinocytes for the presence of HNP-1/2 and were able to purify and sequence it also from these both sources. Our findings clearly indicate that apart from neutrophils also keratinocytes produce the peptide-antibiotics HNP-1 and HNP-2, which might help to keep normal skin healthy.

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Synergistic and Antagonistic Effects of Mutant Peptides Derived From Staphylococcal Enterotoxin A *In Vivo*

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Several bacterial superantigens have recently been shown to be capable of inducing psoriasis in the SCID-hu xenogeneic transplantation model. We were interested in the modulation of this effect by mutated forms of the respective superantigens. Unaffected skin of patients suffering from psoriasis was transplanted onto SCID mice. Subsequently, during a period of 2 wk the grafts were repetitively injected i.c. with 2 μg of the superantigen staphylococcal enterotoxin A (SEA). This regimen resulted in a profound akanthosis. Epidermal thickening was even more pronounced when a mutated form (H187A) of SEA resulting in reduced affinity to MHC class II was coinjected. Another mutated peptide (F47A/D227A) exhibiting no measurable MHC class II affinity was shown to inhibit epidermal thickening triggered by wild-type SEA when injected in a 10-fold higher dose. This effect was specific since induction of psoriasiform epidermal changes by SEB could not be blocked. Since, in contrast to other superantigens, SEA is capable of inducing epidermal thickening but not the typical appearance of psoriasis we conclude that bacterial superantigens may differ with regard to their effects on human skin. Epidermal thickening triggered by SEA was effectively blocked by a mutated form of this superantigen highlighting the potential therapeutic use of mutated superantigens.

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Chimeric Papillomavirus-Like Particles Carrying the Neutralizing HIV1 gp41-Epitope ELDKWAS are Immunogenic

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Papillomavirus-like particles (VLP) are rigid and repetitive structures that induce high-titer neutralizing and protective antibodies against papillomavirus (PV) surface epitopes. Since there is no crystal structure of the PV capsid the exact location of these conformation-dependent epitopes is still unknown. To evaluate these epitopes and the potential of VLP as antigen carrier we inserted the 7 amino-acid (aa) peptide ELDKWAS of the HIV1 gp 41-envelope protein, recognized by the broadly neutralizing monoclonal antibody (mAb) 2F5, into hypervariable regions (putative surface "loops") of the L1 major capsid protein of human PV (HPV)16 or bovine PV (BPV). Chimeric proteins were expressed in insect cells and purified on density gradients. By electron microscopy the majority of the constructs presented as aggregates of pentamers, and one BPV-L1 chimera self-assembled into particles morphologically indistinguishable from wt VLP. Binding patterns for mAbs identified several aa residues as parts of the neutralizing PV epitopes. Antisera raised against native but not denatured particulate preparations inhibited BPV VLP-induced hemagglutination of mouse erythrocytes, neutralized BPV *in vitro* and specifically bound the ELDKWAS-peptide. These findings for the first time identify a region of L1 that allows the surface display of a foreign epitope in the context of a self-assembled VLP and support the potential as an immunogenic vaccine carrier. Chimeric VLP will further our understanding of B-cell activation by pathogenic viruses and the design of new recombinant vaccines.

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Involvement of Desmoglein 1 Gene (DSG1) in Susceptibility to Pemphigus Foliaceus

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To determine if desmoglein 1 (DSG1) gene constitutes a candidate gene for pemphigus foliaceus (PF), the sequence of this gene was analysed in 33 PF patients and in healthy subjects.

Because a polymorphism of exon 11 was evidenced in bovine DSG1 gene, we first sequenced exon 11 PCR products amplified from genomic DNA of the patients.

Four mutations responsible for amino acid substitutions (A→C [position 1555], C→A [position 1570], A→C [position 1660], AAAGA→AGAAA [position 1686–1690]) were identified in six patients (18.2%). These mutations were always heterozygous and associated on the same chromosome as determined by sequence analysis of subcloned PCR products, and therefore forming a variant haplotype. A family study evidenced a mendelian inheritance of the variant haplotype. Detection of mutation 1660 by allele specific PCR in 118 healthy controls and sequence analysis of positive samples allowed to detect the variant haplotype in 12 subjects (10.2%, 1 homozygous) ($p = 0.23$).

Sequencing of the cDNA encoding the extracellular part of Dsg1 in three patients revealed another mutation A→C (silent) at position 809 (exon 7). Since substitution 809 was responsible for a *Fau*I restriction site we determined its frequency by PCR-digestion in patients (87,9%, allelic frequency: 66.7%) and controls (71.4%, allelic frequency: 48.5%) ($p = 0.01$). A 101-bp insertion corresponding to the 3′ extremity of intron 6 was also evidenced at exon 6/exon 7 junction on the cDNA of these three patients. This insertion was responsible for a stop codon and therefore, could lead to a truncated isoform of Dsg1 lacking the transmembrane domain (secretory isoform).

This study demonstrates a polymorphism of the human DSG1 gene which may constitute a susceptibility factor to PF, independently or in association with HLA class II molecules.

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The Shed Ectodomain of Collagen XVII/BP180 is Targeted by Autoantibodies in Different Blistering Skin Diseases

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Collagen XVII, or the 180 kDa bullous pemphigoid antigen-2, is an epidermal adhesion molecule. It exists in two forms, as a full-length transmembrane protein and as a 120 kDa collagenous ectodomain which is shed from the keratinocyte surface by furin mediated proteolysis. The authentic, shed ectodomain of human collagen XVII was characterized as an antigen with domain-specific antibodies against recombinant fragments. It was found in the epidermis, keratinocyte culture media, amniotic fluid and pemphigoid blister fluid. In immunoassays, IgG and IgA autoantibodies in sera or blister fluid of patients with different subepidermal blistering skin diseases recognized the 120 kDa ectodomain. When the autoantibodies were affinity-adsorbed from patient sera with the ectodomain, the IgG and IgA bound to the blister roof of NaCl-split normal skin, at the proximal surface of the epidermis, but not to mutant collagen XVII-deficient skin. The antibody reactivity was not dependent on the native conformation or N-glycosylation of the protein, but was abolished by collagenase-treatment. Epitopes targeted by IgG and IgA in sera of 107 patients with bullous diseases were assessed using full-length collagen XVII, the shed ectodomain and recombinant fragments. In disorders of the pemphigoid group, IgG reactive with the full-length collagen XVII also recognized the 120 kDa soluble ectodomain. In linear IgA dermatosis and chronic bullous dermatosis of childhood, IgA were reactive with the soluble ectodomain. The use of recombinant collagen XVII fragments demonstrated that epitopes were spread over noncollagenous and collagenous domains of the protein. These data identify the authentic, shed ectodomain of collagen XVII as an epidermal autoantigen which is a targeted by IgG and IgA autoantibodies and contains epitopes in several subdomains.

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IgG Auto-Antibodies to BP180 Are Directly Pathogenic in Human Skin and Induce Sub-Epidermal Blisters: Evidence Using Skin Explant Culture and SCID Mouse Grafted with Human Skin

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Previous studies had suggested that anti-BP180 antibodies could induce subepidermal blisters only in the presence of complements and PMN/eosinophils. We here provide evidence that anti-BP180 antibodies are pathogenic with absence of complements or PMN/eosinophils. Anti-BP180 IgG antibodies induced subepidermal blisters both *in vitro* in an skin explant culture system and *in vivo* in SCID mouse grafted with normal human skin. IgG4 deposits were found along the dermal-epidermal junction and localized to the epidermal side of the lesional skin. C3 deposits along the basement membrane zone were insignificant and there were no major eosinophilic infiltrates in the bullous lesions. By immunogold electron microscopy, the IgG antibodies were mainly localized within the lamina lucida. We further sought to determine the mechanism by which the anti-BP180 antibodies may mediate their pathogenic effect by using a keratinocyte culture system and a rabbit antibody to the extracellular part of BP180. Followed by incubation with anti-BP180 antibody, a re-distribution of BP180 from the cell membrane into the cytoplasm was found. This change was accompanied by BP180 phosphorylation. The anti-BP180 effect in cell/matrix dissociation was further inhibited by H7, an inhibitor of protein kinase C (PKC). This finding suggests that the anti-BP180 pathogenic effect is mediated by PKC signaling pathway. Clinically, a correlation between IgG4 anti-BP180 serum titer and disease severity was found in patients with BP ($p < 0.05$). This study demonstrates, for the first time, the direct pathogenic role of anti-BP180 antibodies in human skin.

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Tissue Transglutaminase Antibodies in Dermatitis Herpetiformis

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Tissue transglutaminase has recently been claimed to be the autoantigen in coeliac disease. The incidence of IgA antibodies to tissue transglutaminase has been found to be 95–98% by enzyme-linked immunosorbent assay in untreated coeliac patients, which correlates with the incidence of antiendomysial antibodies in this disease. All patients who had tissue transglutaminase also had endomysial antibodies. We have screened 72 patients with dermatitis herpetiformis to determine if tissue transglutaminase antibodies also occur in this disease and their relationship to gluten withdrawal. Of the 72 patients, 37 were taking a strict gluten free diet, 20 a partial diet and 15 a normal diet. Tissue transglutaminase antibodies were screened for by an enzyme-linked immunosorbent assay and endomysial antibodies by an indirect immunofluorescence method. The results were analysed in relation to small intestinal biopsy findings in 13 of the 15 untreated patients. Of the 37 patients taking a strict gluten free diet, one had tissue transglutaminase antibodies, but none had endomysial antibodies. Of the 20 patients taking a partial diet, five had endomysial and seven had tissue transglutaminase antibodies. Of the 13 patients taking a normal diet, nine had villous atrophy and in eight transglutaminase antibodies were present; two had normal villous architecture but raised intraepithelial lymphocyte counts and both had tissue transglutaminase antibodies, while two with normal villous architecture and intraepithelial lymphocyte counts did not have transglutaminase antibodies. These results show that transglutaminase antibodies are also present in dermatitis herpetiformis, and disappear with gluten withdrawal. However as with the antireticulin and antiendomysial antibodies the incidence in dermatitis herpetiformis is less than that found in coeliac disease and may be related to the less severe enteropathy that occurs in dermatitis herpetiformis.

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Anti-Epiligrin (Laminin 5) Cicatricial Pemphigoid (AECP): An Underdiagnosed Entity Within the Group of Scarring Autoimmune Bullous Diseases

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Scarring lesions of mucous membranes are found in different autoimmune subepidermal blistering diseases characterized by autoantibodies to BP180, α4 integrin, laminin 5, type VII collagen, or other yet unknown autoantigens. A scarring disease with autoantibodies to the α3 subunit of laminin 5 has recently been described as antiepiligrin cicatricial pemphigoid (AECP). Only a few patients with AECP have been reported to date. The aim of the present study was to analyze the relative frequency of AECP among patients with the clinical phenotype of cicatricial pemphigoid (CP). Sera from 16 consecutive patients with the clinical phenotype of CP and linear deposits of autoantibodies at the basement membrane zone as detected by direct immunofluorescence (IF) were analyzed by immunoprecipitation of radiolabeled media and extracts of human keratinocytes. In addition, the sera were studied by immunoblot analysis of cultured keratinocyte extracts, keratinocyte extracellular matrix, epidermal and dermal extracts, and with recombinant forms of N- and C-terminal fragments of the BP180 ectodomain. Nine patients had circulating autoantibodies by indirect IF on NaCl-split skin; patients' IgG bound to the epidermal (n = 2), dermal (n = 5), or both sides (n = 2) of this test substrate. Interestingly, all five patients with dermal binding IgG immunoprecipitated laminin 5 from extracts and media of cultured keratinocytes and four of these sera reacted with the α3 subunit of laminin 5 by immunoblotting. A total of six CP patients, including two indirect IF-negative sera, had autoantibodies to BP180 whereas none of the dermal binding sera reacted with type VII collagen. Our findings suggest that among patients with the clinical phenotype of CP, AECP may represent a significant subset.

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Identification of Desmoglein 1 as Autoantigen in a Patient with Intraepidermal Neutrophilic IgA Dermatosis Type of IgA Pemphigus

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In a 51-year-old female patient with intraepidermal neutrophilic IgA dermatosis (IEN) type of IgA pemphigus, circulating IgA, but not IgG, autoantibodies were detected to bind to the cell surface of the whole epidermis, being much stronger in the upper epidermis. In the patient's skin a heavy intraepidermal IgA staining was observed throughout the whole epidermis, accompanied by a weak IgG and a more prominent C3 staining. IgA from the patient's serum showed no reactivity either with epidermal proteins by immunoblot analysis, or with COS 7 cells transiently transfected with mammalian cell expression constructs containing full length human Dsc1, Dsc2 and Dsc3. Our patient's IgA specifically reacted with conformational epitopes of human desmoglein (Dsg) 1 but not Dsg 3, when studied in a previously established, here modified enzyme-linked immunoabsorbent assay (ELISA) of baculovirus expression system. The immunreactivity against keratinocyte cell surface was completely removed from the serum of the patient by preincubation with recombinant Dsg1 baculoprotein. This finding indicates that the sera possess only IgA antibodies against the extracellular domain of Dsg1 baculoprotein, but no antibodies against components of keratinocyte cell surface other than Dsg1. This is the first case of IgA pemphigus where Dsg1 has been identified as the autoantigen.

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Comparison of a Tissue Transglutaminase Enzyme-Linked Immunosorbent Assay with the Endomysium Antibody Test in the Diagnosis of Dermatitis Herpetiformis

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Tissue transglutaminase (tTG) has recently been suggested by Dieterich *et al*. to be the autoantigen of coeliac disease against which endomysium antibodies (EMA) are directed. Preliminary studies showed that tTG may be also the autoantigen of EMA positive patients with dermatitis herpetiformis (DH). We investigated whether this finding can be used for diagnostic purposes in a tTG-based enzyme-linked immunosorbent assay (ELISA) also for DH. Serum samples from DH patients and controls were examined for tTG autoantibodies of IgA type using microtiter plates coated with tTG, incubating with the patients' sera in different dilutions, and detecting bound IgA by peroxidase-conjugated antihuman IgA immunoglobulins. Results of tTG ELISA were compared to the results of the routine EMA test using monkey oesophagus sections. Serum IgA antibodies of patients with dermatitis herpetiformis react with the tTG also when the patients do not complain of symptoms of gluten sensitive enteropathy. The tTG-based ELISA seems to be a suitable method for diagnosis of gluten sensitive enteropathy in patients with DH.

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Are Infiltrating T cells Involved in the Pathogenesis of Pemphigus?

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Recent studies showed that Dsg-3 specific T-cells lines and clones express a CD4+ T-cell phenotype and are able, after stimulation, to secrete IL-4 and IL-6.

To further investigate the involvement of T-lymphocytes, their related cytokines and dendritic cells in the pathogenesis of pemphigus, we evaluated the *in vivo* expression of a large mAb panel (anti-CD3, -CD4, -CD8, -CD25, -CD30, -CD1a, -CD40, -CD40L, -IL-2, -IL-4, -IL-6, -IL-7, -IL-13, -IFN-γ) using an APAAP procedure. We than compared the results obtained from lesional skin of six pemphigus patients (three pemphigus vulgaris and three pemphigus foliaceus) with those from uninvolved skin of the same patients and of healthy controls.

The number of infiltrating CD3+ T cells appeared considerably decreased in uninvolved skin (14.7 sd ± 9.9 for field) if compared with lesional skin (61.8 sd ± 59.1 for field), while CD4+ T cells did not show significant differences. These CD4+ T cells showed signs of activation (CD25+ and CD30+) only in lesional skin. We also documented an important decrease of CD1a+ cells in uninvolved skin and a further increase of these cells in lesional skin. The CD40L expression was detected only in lesional skin, while CD40 was equally expressed in lesional and uninvolved skin.

The cytokine analysis of lesional skin revealed a positive intense staining for IL-2 and IL-4, and a weak/focal staining for IL-6, IL-7, IL-13 and INF-γ. Uninvolved skin didn't show any significant staining for the above mentioned cytokines except for a moderate expression for IL-2.

These findings indicate the involvement of a Th0/Th2-like response that seems important in the blister formation of pemphigus.

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The Use of Urine in the Diagnosis of the Autoimmune Blistering Diseases

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In the subepidermal bullous diseases autoantibodies to the basement membrane zone (BMZ) are found in skin, blood and blister fluid. Venepuncture can be difficult in children and the elderly, and blister fluid is not always available. Urine is readily available and easily collected. We have investigated the use of urine for the diagnosis of immunobullous disease by indirect immunofluoresence (IMF).

Urine and serum were collected at the same time from patients with bullous pemphigoid (BP) (no. 43), cicatricial pemphigoid (CP) (no 8) and linear IgA disease (LAD) (no 9). Urine and serum samples were frozen and stored at −20°C until required for IMF on intact and salt split skin. Urine was concentrated by ultrafiltration and ultracentrifugation and serum diluted to determine the titre of BMZ antibodies. Urine from five, and serum from 10, volunteers acted as the negative controls.

IgG subclass analysis was carried out on 14 positive urines and corresponding serum samples. Immunoblotting of epidermal tissue extracts using concentrated urine and corresponding serum samples from 20 patients was done to determine whether IgG BMZ antibodies in these two fluids recognize the same target antigens.

Our results show that IgG BMZ antibodies can be detected in the urine of many patients with BP and some with CP. There was a direct correlation between the detection of autoantibodies in the urine and the serum antibody titre. The threshold for the appearance of antibodies in the urine was a high titre of circulating antibodies (>1:160). The subclass distribution was similar but not identical in urine and serum, IgG4 was the main subclass detected, followed by IgG1, IgG2 was rarely detected, and IgG3 was not detected.

We have shown that BMZ antibodies are present in the urine and target BP230 and BP180.

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Suprabasal Keratinocytes Express HLA Class II Antigens in Cicatricial Pemphigoid

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HLA class II molecule expression is usually restricted to Langerhans cells in normal epidermis. In some pathological conditions including autoimmunity, hyperexpression of HLA class II molecules has been reported in cells usually deprived of this capacity. In previous studies, it has been shown that keratinocytes do not express HLA class II antigens in bullous pemphigoid. Considering that bullous and cicatricial pemphigoid are different disease regarding HLA phenotypes of the patients and immunoultrastructural findings, the aim of the present study was to evaluate HLA expression in bullous and cicatricial pemphigoid and to correlate it to ultrastructural patterns. A retrospective study was performed using frozen sections from skin biopsies of patients with bullous (N = 9) ans cicatricial pemphigoid (N = 9). A double staining procedure was used : the first step was to identify Langerhans cells by their immunoreactivity with a horseradish labelled anti CD1a monoclonal antibody (Dako France). During the second step frozen sections were incubated with either an anti HLA DR or an anti-HLA DQ monoclonal antibody using APAAP (Biocell). All patients were diagnosed using clinical and immunopathological findings. A biopsy for immunoelectron microscopy was performed in all patiens showing deposits restricted to the upper lamina lucida in patients with bullous pemphigoid and deposits overflowing the lamina densa in cicatricial pemphigoid. Six frozen skin biopsies of patients without bullous disorder were used as controls. Langerhans cells were clearly differentiated from keratinocytes by the double labelling with anti CD1a and anti HLA DR or DQ. Skin sections from the nine patients with bullous pemphigoid did not show any labelling of the keratinocytes with either anti HLA DR or HLA DQ. On the other hand skin sections from patients with cicatricial pemphigoid showed a labelling of some keratinocytes with HLA DR and HLA DQ. The labelled keratinocytes were located closely to the basement membrane and usually formed some clusters. Controls remained negative. This study confirms the previous findings in bullous pemphigoid and suggest that in cicatricial pemphigoid some suprabasal keratinocytes can express HLA class II antigens The question remain to know whether this expression is a primary phenomenon or secondary to interferon gamma production by activated T lymphocytes which has been reported in cicatricial pemphigoid.

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Eosinophils and Pemphigus

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Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are autoimmune blistering diseases in which infiltrating cells, in particular eosinophils, seem to contribute to the blister formation. Histologic studies have demonstrated the presence of eosinophils in early and late pemphigus lesions in both the dermis, as part of the cellular infiltrate, and in the epidermis (eosinophilic spongiosis). In order to evaluate the role of eosinophilic inflammation in these diseases and to detect the presence and activity of eosinophils, we studied the immunoreactive deposition (EG1 and EG2) of the Eosinophil Cationic Protein (ECP), an eosinophil derived protein, in lesional and uninvolved skin of six pemphigus patients (three PV and three PF). We also analysed the expression of their tightly related cytokine interleukin (IL)-5 in the same sites. In addition we compared the ECP serum levels of 18 pemphigus patients (14 PV and four PF) with those of 30 healthy subjects using a double antibody radioimmunoassay (ECP/RIA) method.
A positive staining for EG1 and EG2 was detected in lesional skin of PV and PF patients in both dermal (perivascular and scattered distribution) and epidermal sites. The number of EG2+ cells appeared increased in PF patients (25.8 sd ± 9.01) if compared with PV patients (23.1 sd ± 11.54). In contrast the staining for EG2 in uninvolved skin was usually negative. An intense staining for IL-5 was detected in both varieties. IL-5 was present mainly in vascular and perivascular sites of the superficial dermis, and in two cases, it was also expressed at the dermo-epidermal junction. The serum levels of ECP (average 30 ng per ml) appeared increased in our 18 pemphigus patients if compared to the serum levels detected in healthy control subjects (average 4.5 ng per ml).
In conclusion these data suggest a relevant participation of eosinophils in causing tissue damage in pemphigus.

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Rapid Response of IgA Pemphigus of the Subcorneal Pustular Dermatosis Type to Treatment with Isotretinoin

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The diagnosis of IgA pemphigus and its two subtypes, intraepidermal neutrophilic IgA dermatosis (IgA pemphigus vulgaris) and subcorneal pustular dermatosis (IgA pemphigus foliaceus) is important because of the different therapeutic management they require. Sometimes, these dermatoses fail to respond to all currently known therapeutic modalities. We present a patient with the subcorneal pustular dermatosis type of IgA pemphigus who rapidly responded to systemic treatment with isotretinoin. The diagnosis was established by detecting IgA serum reactivity to unfixed COS7 cells transfected with desmocollin 1 by indirect immunofluorescence microscopy. In contrast, no IgA or IgG reactivity was found to recombinant forms of desmoglein 1 and 3 by ELISA. The disease was not effectively controlled by systemic steroids and dapson treatment had to be discontinued because of severe side-effects. Treatment with oral isotretinoin 20 mg daily lead to a striking improvement of skin lesions within 4 d and complete clearance within 1 wk. In general, the specific binding of human IgA is thought to provide resistance to protease digestion, thus allowing for efficient binding of neutrophils. Isotretinoin may interrupt this pathogenetic process by inhibition of neutrophil chemotaxis across biologic barriers thereby repressing accumulation of neutrophils and their destructive actions at the antibody binding site. Our data demonstrate that isotretinoin is an effective drug in treatment of IgA pemphigus foliaceus. This observation also suggests that isotretinoin might be of therapeutic value in other conditions with pronounced involvement of neutrophils.

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Trace Element Determination in Human Nails, During Corticotherapy in Pemphigus Patients

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We studied the trace elements contained in human nails by means of the electronic microprobe. This technique was based on the emission of X-ray through electronic excitation. It traced 9–11 elements in human nail samples, from both hands and feet of five patients with pemphigus vulgaris which were under corticotherapy for a long period of time (12 mo to 10 y). The determination was repeated 2–3 times in the same patient. The major element faced was S (sulfur), with a mean value of 40 000 p.p.m. (parts per million). Ca (calcium), Cl (clor), K (potassium) had values between large ranges (230–12,000 ppm), influenced by factors like sex, age, nutrition, environment and especially by corticotherapy. Other elements traced by this technique were Si (silicium), Mn (manganese), Cu (copper), and Fe (iron). Mg (magnesium), Al (aluminum), Zn (zinc), were absent. The electronic microprobe didn't trace Na (sodium) in any of the nail samples. In our determination, during corticotherapy, S content of human nails increased.

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A Case of Pemphigus Erythematosus Displaying Autoantibody Reactivity with both Pemphigus Foliaceus and Bullous Pemphigoid Antigens

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We present the case of a 63-y-old Caucasian who visited our department in March 1999 complaining of a single erythematous lesion with erosions and crusts on the forehead. The lesion has appeared several months earlier and was treated unsuccessfully with topical antibiotic and anti-inflammatory drugs. The remaining skin and mucous membranes were uninvolved. Results of the laboratory investigations were negative or within normal limits. Antinuclear antibodies and subsets were undetectable. Chest X-ray and sonography of abdominal organs were normal except for mild hepatomegalia. A biopsy specimen taken from the lesion showed both suprabasal blister formation with a tombstone pattern in addition to superficial intraepidermal split formation. A dense perivascular infiltrate consisting of lymphocytes, plasma cells and eosinophils was present. Based on the clinical and the histopathologic findings a diagnosis of pemphigus erythematosus was established. Direct immunofluorescence of perilesional skin showed deposition of IgG at the cell surface. Deposition of C3 was seen at the cell surface in the epidermis and at the BMZ. In the serum, indirect immunofluorescence on monkey esophagus detected anti cell surface antibodies of IgG class at a titer of 1:40. Indirect immunofluorescence on sections of human split skin separated within the lamina lucida, revealed serum antibodies of the IgG class also reactive with the epidermal side of the split at a titer of 1:40. Immunoblot and immunoprecipitation studies were performed using normal human epidermal extracts. By immunoblotting, a band migrating at 230 kDa representing the 230 kDa bullous pemphigoid antigen was present in addition to the 160 kDa pemphigus foliaceus antigen. By immunoprecipitation the patient's serum reacted with the 230 kDa bullous pemphigoid antigen, only. Thus, our data show that the patient displays a "mixed bullous disease phenotype" with autoantibodies directed against PF and BP antigens.

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Fluorescence Overlay Antigen Mapping and Laser Scanning Confocal Microscopy in Differentiation Cicatricial Pemphigoid and Epidermolysis Bullosa Acquisita

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Two patients fulfilling clinical, immunological and immunoelectron microscopic criteria for cicatricial pemphigoid (CP) using fluorescence overlay antigen mapping (FOAM) and laser scanning confocal microscopy (LSCM) techniques were studied. FOAM and LSCM were performed on the biopsies taken from patients' uninvolved skin using rabbit polyclonal antibody NC2–10 against carboxy-terminal portion of collagen VII or monoclonal anti laminin-1 antibody labeled with rhodamin, whereas *in vivo* bound immunoglobulins were labeled with fluorescein. In both cases the fluorescence overlay image showed a distinctive red fluorescence of collagen VII beneath the yellow-orange band of immunodeposits, which was also confirmed by LSCM observation. FOAM with antilaminin-1 showed in both cases a yellow linear reaction along the basement membrane. LSCM examinations confirmed a complete overlap of green fluorescence of *in vivo* bound deposits and red fluorescence of laminin-1 in one case, whereas in second patient laminin-1 was localized on the epidermal side of *in vivo* bound IgG. In contrast to CP, FOAM performed on biopsy taken from epidermolysis bullosa acquisita (EBA) patient using anticollagen VII antibody showed yellow reaction due to overlap collagen VII and *in vivo* bound IgG. Both FOAM and LSCM techniques could be used for differentiation CP and EBA, but LSCM allows for more precise localization of *in vivo* bound deposits than FOAM and may be of special value for differentiation of CP from bullous pemphigoid or may even allows for the distinction of different subtypes of CP.

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Immunocytochemical Study of Desmosomal Proteins in Pemphigus Vulgaris and Hailey-Hailey Disease

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The aim of the study was to compare the mechanisms of desmosomes detachment in autoimmune and nonimmune acantholysis. Expression and distribution of desmosomal proteins (Ds) in acantholytic cells of five patients with pemphigus vulgaris (PV) and three patients with Hailey-Hailey disease (HHD) were studied with the use of following immunocytochemical techniques: immunofluorescence (IF), fluorescence overlay antigen mapping (FOAM), laser scanning confocal microscopy (LSCM), and postembedding immunogold electron microscopy (IEM). IF and LSCM showed staining of Ds proteins in the peripheral cytoplasm: granular – in early lesions, and diffuse – in advanced acantholysis of PV. FOAM and LSCM studies, with the use of double labeling of DS proteins and antihuman IgG, revealed that desmoglein 1, desmocollin 1and plakoglobin clusters colocalized with auto-antibodies and were internalized. IEM showed disruption of Ds at their extracellular parts, followed by endocytosis of half desmosomes. In contrast to PV, acantholytic cells of nonimmune mediated Hailey-Hailey disease (HHD) revealed perinuclear localization of Ds proteins and their attachment to retracted KIF. Our study showed that desmosomes in PV before undergoing endocytosis are split in the extracellular space. These observations favor the hypothesis that binding of PV antibodies to Ds proteins is responsible for acantholysis by modification of adhesive properties of Ds proteins resulting in desmosome dissociation. Enzymatic degradation of split desmosomes appears to be a secondary phenomenon in the process of acantholysis.

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Deficiency in collagen type III is a well recognised cause of vascular fragility in Ehlers Danlos Syndrome type IV, other symptoms of which range from thin skin, premature ageing of the extremities (acrogeria), short stature and pneumoperitoneal and intestinal fragility to a more subtle external phenotype resembling EDS type III/Benign Hypermobile Syndrome (1). All the subtypes are prone to vascular fragility. Collagen type III deficiency results from mutations in the COL3A1 gene. The subtypes in which acrogeria is seen are frequently caused by C-terminal glycine substitutions and exon skips (2), however, exon skips also produce a more variable phenotype (1,2). Here we describe an unusual two generation British family in which the proband died of multiple arterial aneurysms. All the affected family members, including the proband, exhibit multiple premature keloid scars and finger contractures and the facial phenotype of EDS type IV. The proband's affected elder brother requires partial amputation for his finger contractures, and required a 14 pint blood transfusion for the repair of his varicose veins. The proband's affected 12-y-old daughter also has congenital pulmonary stenosis and her affected 13-y-old niece (daughter of the affected elder brother) has cutaneous thinning. Protein analysis shows reduced procollagen and collagen synthesis without over modification of the cell layer protein. Direct sequencing of COL3A1 cDNA, amplified in five overlapping fragments, shows a 54-bp deletion of exon 24. We have two other exon 24 deletions with different clinical phenotypes (1,3) which also differ from those of other published data (4) suggesting that exon 24 is a mutational hot-spot with variable clinical phenotype. Furthermore, the combination of severe keloidal scarring and premature finger contractures is unusual, implying a dysregulation of collagen synthesis mediated by the abnormal exon deletion. References: 1. Pope FM, Narcisi P, Nicholls AC *et al*: *Br J Dermatol* 135:231–236, 1996; 2. Pope FM, Burrows NP: *J Med Genetics* 34:400–410, 1997; 3. Schwarze U, Goldstein JA, Byers PH: *Am J Human Genetics* 61:1276–1286, 1997.

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Two Transcripts of the COL17A1 Gene do not Explain the Presence of Two Forms of Collagen XVII Protein

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BP180/collagen XVII, a structural component of hemidesmosomes exists in two forms, a 180 kDa type II transmembrane protein, obtained from keratinocytes and a 120 kDa soluble form, found in culture media of keratinocytes. We were interested in the mechanisms of the generation of the short form and therefore performed Northern blot analysis and RACE experiments. Hybridization with probes specific for either the 5′ end or the 3′ end of the collagen XVII cDNA identified two transcripts of about 6,0 kb and 5,4 kb 3′ RACE experiments revealed two types of cDNA for the 3'UTR region of collagen XVII mRNA. DNA-sequence analysis showed the collagen XVII specificity of both cDNA types and revealed a 607 bp deletion in the shorter form. RT-PCR experiments using specific 3′ end primers verified these results. Amplification of genomic DNA using identical primers resulted only in one specific product, corresponding to the longer cDNA suggesting that both cDNAs are products of the same collagen XVII gene, COL17A1.

In summary, we identified a second mRNA for collagen XVII. This mRNA contained a 607 bp deletion in 3′ UTR region. However the deletion is not located within the ORF, and therefore, the second mRNA does not correspond to the shorter form of collagen XVII, the 120 kDa soluble ectodomain. These results are in concert with the assumption that the soluble ectodomain of collagen XVII is generated by specific proteolytic processing rather than by alternative splicing. Nevertheless, the function of a second collagen XVII mRNA is of interest with regard to regulation of collagen XVII expression.

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Abstract withdrawn

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Differential Regulation of Bcl-2 and Bax in Apoptosis of Primary Fibroblasts Cultured in a Contractile Collagenous Matrix

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Primary human fibroblasts are susceptible to apoptosis, when cultured in a contractile three-dimensional collagenous matrix. Furthermore, reactive oxygen species are produced during this process, indicating that mitochondria are involved. Therefore, in this study we investigated the role of members of the bcl-2 family in fibroblasts undergoing apoptosis in contractile collagen matrices.

Primary human fibroblasts were cultured in three-dimensional contractile or anchored collagen matrices. At distinct time points apoptosis-specific cytoplasmic histone-associated DNA fragments were quantified by a sandwich Elisa. In parallel RT-PCR and Western blot analyses were performed. Bcl-2 protein was markedly reduced in apoptotic cells, whereas Bax levels remained unaltered in contractile collagenous matrices. In anchored gels, where no apoptosis was detectable, the levels of both proteins remained unaltered.

These results are in accordance with data obtained from analogous experiments using RT-PCR. They support the hypothesis, that members of the bcl-2 family are intimately involved in apoptosis of fibroblasts, and that the proportion of Bcl-2 and Bax reflects the apoptotic status of fibroblasts cultured in our system. Since there is also a regulation on the mRNA-level, RT-PCR of bcl-2 and bax can be used as an indirect apoptosis assay, which is relatively easy to perform in comparison to the quantitation of histone-associated low molecular weight DNA.

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Alteration of the Helical Collagen Structure in Patients with Hypermobility Syndrome and Ehlers–Danlos Syndrome: *In Vivo* Studies by Raman Spectroscopy

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Mutations in collagen genes in Ehlers–Danlos syndrome (ED) result in collagen fiber alterations leading to connective tissue malfunction. Little is known about the structure of collagen in the skin in hypermobility syndrome (HS) which is related to ED. Near infrared Fourier transform Raman spectroscopy (NIR-FT Raman spectroscopy) is a non destructive method recently used for protein, lipid and water structure analysis in intact normal human skin.[1] The position of the spectral bands reflecting vibrations of protein bonds (amide I and III) allow to describe protein secondary structure such as helix, sheet or coil. Here, we looked into collagen structure in patients with clinically diagnosed HS (seven patients), ED (three patients) and 24 healthy volunteers using NIR-FT Raman spectroscopy. In the spectra obtained via optic fibers directly from the skin surface, the ratio between areas under amide III band reflecting helical structure (1270 cm^{-1}) and amide III showing random coil (1240 cm^{-1}) was calculated. The ratio was 2.1 for the HS and ED patients while 1.4 for healthy volunteers. The half width (full width at half height) of the helix band was nearly a factor of 2 larger for HS and ED patients as compared to results for healthy volunteers. This shows that the helical part of the collagen is more disordered for HS and ED patients. Moreover, electron microscopy of skin biopsies from HS and ED patients showed disorganised, twisted, angular collagen fibers. Thus, structural alterations in dermal collagen is found not only in ED but also in HS which confirms a close relationship between these two diseases.

1. M. Gniadecka *et al*: *J Investigative Dermatology*, 111:1129, 1998

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The Novel Basement Membrane Antigen of MoAb LH24 – is it Collagen VII?

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The MoAb LH24 was raised against basement membrane zone (BMZ) extracts. It has been characterised using indirect immunofluorescence on intact and salt split skin; skin treated with the proteases: trypsin, pepsin, pronase, dispase, elastase and collagenase; skin treated with heat (56°C) and EDTA. The distribution of the antigen in normal human skin and mucosa and the expression of the antigen by cultured human keratinocytes, fibroblasts, trypsinised epidermal cells, and cylindroma tissue was studied. The results were compared with MoAbs and polyclonal antibodies to collagen VII (LH7.2, L3d, polyclonal anti-collagen VII, and EBA sera) and laminin 5 (GB3 and BM160). Attempts to further characterise the antigen by immunoblotting and immunogold electron microscopy were unsuccessful.

LH24 bound to the dermal aspect of salt split skin, and to the epidermal BMZ of normal human skin, oral and vaginal mucosa. The pattern observed on cylindroma was identical to collagen VII, and there was no resemblance to the pattern of expression of laminin 5 on cultured keratinocytes. The target antigen was heat labile compared to collagen VII (destroyed by 30 s at 56°C, collagen VII present after 150 s). The LH24 antigen was remarkably stable compared to laminin 5 and collagen VII, as it was not destroyed by trypsin, pepsin, pronase, dispase, and elastase thus clearly distinguishing it from laminin 5 and the NC1 domain of collagen VII. It was partially collagenase sensitive. The preservation of reactivity with pronase, sensitivity to heat, and double labelling suggest LH24 antigen may not be the triple helix of collagen VII.

These results suggest that the LH24 target antigen may be a novel dermal protein of the BMZ. LH24 antigen may be a candidate gene for genetic blistering disorders and a target antigen for immunobullous diseases.

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Modulation of Skin Collagen Metabolism by Irradiation: Collagen Synthesis is Increased in Human Skin

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The aim of this study was to analyze the effect of therapeutic irradiation on human skin collagen synthesis, skin thickness and collagen degradation markers. Twenty women who had been treated for breast cancer with operation and radiation therapy were included in the study.

Suction blisters were induced on the irradiated skin area and the corresponding skin area of the healthy breast. Suction blister fluid and serum samples were analyzed for procollagen propeptides PINP and PIIINP, tissue inhibitors of matrix metalloproteinases TIMP-1 and TIMP-2 and matrix metalloproteinases MMP-9 and MMP-2/TIMP-2 complex. Bunch biopsies from irradiated skin and corresponding healthy skin area were analyzed for PINP and PIIINP.

In treated skin, skin thickness, the number of PINP positive fibroblasts and PINP, PIIINP, TIMP-1 and MMP-2/TIMP-2 complex levels in SBF were significantly higher in comparison with nontreated skin. The levels of TIMP-2 were similar in treated and nontreated skin. MMP-9 level in SBF was below the detection level of the assay used. The serum levels of PINP, PIIINP, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex were not significantly affected. MMP-9 level in serum samples was higher in subjects than the reference values.

We conclude that increased collagen synthesis and increased accumulation of connective tissue in irradiated skin has now been firmly shown and this gives an opportunity to treat this condition. Especially topical glucocorticoids would be beneficial, since they efficiently downregulate collagen synthesis.

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Fibroblast–Collagen Interaction and Signal Transduction

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Interaction of fibroblasts with the surrounding extracellular matrix is an important control element regulating matrix deposition in wound healing and fibrosis. We have previously shown that this interaction is mainly mediated via the $\alpha 2\beta 1$ and the $\alpha 1\beta 1$ integrin receptors which generate specific signals influencing gene expression in fibroblasts. The $\alpha 2\beta 1$ mediates signals to upregulate metalloproteinase expression at the transcriptional level, involving activation of MAP-kinases. Independent experimental data indicated that activation of $\alpha 1\beta 1$ receptors controls downregulation of collagen I gene expression. It became clear that MMP induction and collagen regulation involve different intracellular signal transduction pathways.

In order to further understand the $\alpha 1\beta 1$-elicited transduction pathway, we started a yeast two-hybrid screen aiming at identifying proteins that bind to the intracellular domain of the $\alpha 1$ chain. Some of the proteins thus identified have previously been described in different contexts. Others, however, represent unknown sequences of unknown function. Some of these have an affinity for different integrin α-chains, and most of these were found to bind to the conserved region of the α-cytodomains. Others, however, were found to interact with $\alpha 1$ integrin exclusively. Of these, some were further characterized; they display coiled-coil and proline-rich domains, indicating their potential involvement in protein–protein interactions. The tissue and cellular distribution of selected proteins, and their functions in biological and pathological processes, involving deposition of extacellular matrix, are being determined.

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Collagen Cross-Linking in Various Sclerotic Diseases

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Proper formation of collagen cross-links is presumbly essential for the biomechanical stability of organs. These cross-links could be generated via two different pathways: lysine-aldol (lys-ald) and hydroxylysine-aldol (hyl-ald). In normal skin, cross-links are generated via the lys-ald-pathway (Histidinohydroxylysinonorleucine, HHL; Histidino-hydroxymerodesmosine, HHMD), whereas in sclerotic skin of lipodermatosclerosis (LDS) an increase in hyl-ald derived cross-link hydroxylysyl-pyridinoline (HP) and lysyl-pyridinoline (LP) is observed. In this study we analyzed mature cross-links of LDS ($n = 7$) and morphea ($n = 9$) to address the question, whether the increase in HP is due to a selective increase of hyl-ald derived cross-links or to a general increase of all cross-links in skin.

Both, LDS and morphea showed an increase in HP and LP. Furthermore, in morphea elevated levels of lys-ald derived cross-links were observed (HHL + HHMD), whereas LDS showed a decrease of these cross-links and of total cross-links as well.

The cross-link pattern of sclerotic skin of morphea can be characterized by a general increase of collagen cross-links, which may be due to an inceased expression of lysylhydroxylase and lysyloxidase. This is in contrast to the situation in LDS, where only an increase in hyl-ald derived cross-links HP and LP is observed, together with a decrease in lys-ald derived cross-links HHL and HHMD. In summary, our study shows that cross-linking of collagen in sclerotic skin diseases is regulated in different ways and emphasizes the importend role of HP-cross-links for hardening of tissue.

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Stem Cell Factor Levels in Plasma and in Fibroblast Cultures in Patients with Systemic Sclerosis

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Stem cell factor (SCF) is a pleiotropic growth factor with diverse hematopoietic target cells, including early progenitor cells. Fibroblasts are recognised as a source of this cytokine. SCF can stimulate proliferation of mast cells which are postulated to be involved in the development of fibrosis in systemic sclerosis (SSc). The aim of our study was to evaluate SCF levels in both blood plasma and fibroblast cultures obtained from skin of patients with SSc in comparison to healthy controls. The study was performed in 12 patients with SSc (eight with the limited form – lSSc and four with the diffuse one – dSSc), aged 31–70 y, duration of the disease ranged from 1 to 13 y (five patients up to 3 y). The control group was age and sex matched. Skin biopsies were obtained from the forearms, clinically devoid of sclerotic changes. Fibroblast cultures were established from skin punch biopsies treated with enzymes (collagenase and deoxyribonuclease) and cultured in modified Eagle's medium. SCF levels were measured by Elisa method (Quantikine R and D Systems) in the fibroblast culture supernatant of the first and second passage and in plasma of the patients

SCF levels in plasma of the SSc patients ranged from 1251.8 to 3039.9 pg per ml (mean 1625.7) whereas in the control group from 1193.0 to 2222.4 pg per ml (mean 1639.5). The difference at $\alpha = 0.05$ between those two groups was statistically insignificant. There was also no statistically significant difference between the patients with lSSc and dSSc. In supernatants from the fibroblast of the first passage (counted for 100 000 fibroblasts), SCF levels ranged from: (a) 5.2–20.2 pg per ml (mean 10.86) in SSc group; (b) 3.0–20.5 pg per ml (mean 13.7) in control group. The differences were statistically insignificant. However, in supernatants from fibroblast of the second passage both in SSc and control group, SCF levels were undetectable despite quite high fibroblast number (up to 400 000 cells).

The obtained results suggest that stimulation of mast cells via SCF originating from fibroblasts seem not to be of prime importance in the development of fibrosis in SSc. It is however, interesting that fibroblasts of the second passage are probably unable to synthesise stem cell factor.

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Retinoic Acid Modulates Fibrillin Expression in Photoaged Skin

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Photoaged skin exhibits coarse and fine wrinkles. The mechanisms of wrinkle formation are unknown but involve changes within the matrix of the dermis and at the dermal-epidermal junction (DEJ) which include reductions in the number of fibrillin microfibrils at the DEJ. Topical all-*trans* retinoic acid (RA) repairs the dermal matrix of photoaged skin after 6 mo. A short-term screening assay for the utility of repair agents, i.e., RA, is desirable. We describe a 4-d screening assay on photoaged skin using fibrillin-1 as the marker for outcome of repair.

Clinically assessed, severely photoaged individuals were recruited ($n = 8$). 15 μl of 0.025% RA in vehicle; 5% sodium lauryl sulphate (SLS, as an irritant control) or vehicle alone were applied under occlusion to photoaged extensor forearm. A fourth control area was also occluded. After 96 h, 4 mm punch biopsies were taken under local anaesthesia, snap frozen and stored at –70°C. Frozen sections (7 μm) were prepared for immunohistochemistry (IHC) and *in situ* hybridization (ISH). IHC was undertaken using a monoclonal antibody to fibrillin-1 and visualised using standard immunoperoxidase techniques. Sections were randomised, blinded and scored for labelling along the DEJ on a 5 point ordinal scale. ISH for FBN-1 mRNA used a biotin-labelled oligonucleotide probe, visualised using standard methods. The numbers of positive cells per high power field (HPF; ×40) were counted for both dermis and epidermis. To account for the effects of RA and SLS on epidermal thickness, epidermal results were corrected for this factor. Mean staining for fibrillin-1 at the DEJ was as follows (mean ± SEM): control: 0.75 ± 0.06, vehicle: 0.49 ± 0.05, SLS: 0.91 ± 0.08, RA: 1.20 ± 0.12. Both RA and SLS were significantly increased compared to control and vehicle groups ($p < 0.05$), with RA treatment significantly increasing fibrillin content over that of SLS ($p < 0.001$). Mean numbers of positive nuclei per HPF in the epidermis (E) and dermis (D) are as follows: control: 36.8 ± 4.26 (E) 18.0 ± 2.05 (D), vehicle: 36.9 ± 4.17 (E) 15.8 ± 2.03 (D), SLS: 54.4 ± 7.04 (E) 28.9 ± 3.70 (D), RA: 73.2 ± 7.74 (E) 19.6 ± 2.50 (D). RA-treatment produced a highly significant increase in FBN-1 mRNA in epidermal keratinocytes ($p < 0.001$) over SLS-treatment ($p < 0.05$).

This study indicates that fibrillin (protein and mRNA) can be used as a "reporter" molecule in this short-term assay of utility of topical agents in the repair of photoaged skin.

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Induction of Matrixmetalloproteinase Expression in Human Dermal Fibroblasts by Hyperosmolaric Stress

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Osmoticaly active substances such as hypertonic carbohydrate or saline solutions are frequently used in the management of chronic leg ulcers because they are well known to improve wound healing. The precise cellular and molecular mechanisms underlying their therapeutic efficacy, however, are poorly understood. In this regard it is of interest that within recent years matrixmetalloproteinase (MMP) induced proteolysis has been recognized as being central to woundhealing. In the present study we have therefore assessed whether hyperosmolaric stress is capable of inducing MMP expression. Cultured human dermal fibroblasts were stimulated with hyperosmolaric concentrations of NaCl, Glucose and Mannitol and subsequently MMP-1 expression analyzed in these cells at the mRNA and protein level. We have observed that treatment of fibroblasts with hyperosmolaric solutions of NaCl, Glucose and Mannitol at concentrations, which did not affect cell viability (trypan blue exclusion, β-actin expression) significantly increased steady-state-levels of MMP-1 mRNA expression (differential RT-PCR) in a dose-and time-dependent manner. In cells treated with 3% NaCl, upregulation of MMP-1 mRNA expression by a factor of 3 was observed 24 h after stimulation. These observations were corroborated and extended by Western blot analysis. Accordingly, hypertonic treatment also significantly induced MMP-1 protein expression in cell lysates prepared from osmotically stressed fibroblasts. These studies for the first time demonstrate that MMP-1 expression can be regulated in human dermal fibroblasts by osmotic stress. This mechanism may be operative if ulcers are treated with hypertonic solutions.

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Avocado Unsaponifiables (AU) Enhance Transforming Growth Factor-β1 (TGF-β1) and Collagen Expression in Cultured Human Dermal Fibroblasts

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Avocado and soya unsaponifiables (ASU) are composed of unsaponifiable fractions of avocado and soybean oils at a ratio of one to two-thirds, respectively. ASU have been reported to stimulate deposition and repair of extracellular matrix components, but the mechanisms underlying their action are not well understood. Considering the biological properties of TGF-β1, which appears to be one of the most potent activators of matrix gene expression in fibroblasts, it was of interest to investigate the effect of the unsaponifiable avocado fractions (AU), called PIA, H and I, on both TGF-β1 and collagen syntheses in human dermal fibroblasts. We demonstrated that a 24-h incubation with 20 μg per ml of AU fractions stimulated collagen synthesis by cultured fibroblasts. This increased expression of collagen was specific, since the level of noncollagenous proteins did not significantly change under the same treatment. Moreover, since some retinoids have been implicated in several biological processes such as proliferation, differentiation, morphogenesis and tissue repair, specially in skin damaged by physical and chemical agents, we investigated the effect of retinol on collagen expression. Therefore, in a second set of experiments, we showed that collagen production was enhanced by exposure to retinol (10^{-5} M and 10^{-8} M). In addition, by measurement of immunodetectable TGF-β1 protein, we demonstrated that TGF-β1 synthesis was enhanced by a 24-h exposure to 20 μg per ml of AU fractions. Interestingly, treatment of fibroblasts by retinol was accompanied by an augmented level of TGF-β1 protein production. We can therefore hypothesize that AU fractions and retinol could enhance collagen biosynthesis through TGF-β1-dependent mechanisms.

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Increased Deposition of Crosslinked Type I Collagen Telopeptides in Lichen Sclerosus et Atrophicus

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The pathogenesis of lichen sclerosus et atrophicus (LSA) is still poorly understood. Histologically LSA is characterized by edema and homogenization of collagen in the upper dermis directly beneath the basement zone. Electron microscopic studies have shown collagen fibrils with different diameters, collagen degeneration and disintegration. The biochemical changes underlying these observations have not yet been well characterized. We have used polyclonal antibodies raised against the mature crosslinked type I collagen telopeptide (ICTP) generated through trypsin digestion of bone type I collagen. Immunohistochemistry was performed in normal skin, LSA and other fibrotic disorders (keloid, scar tissue) as a control. In normal skin, type I collagen telopeptide could only be detected in the reticular dermis. In contrast in LSA, marked staining for type I collagen telopeptide was detected just below the basement membrane zone and in the area of collagen homogenization. As ICTP is readily digested by pepsin, loss of staining after prior pepsin digestion further confirmed the specificity of the markedly increased detection of ICTP in LSA. In contrast, keloid and scar tissue were characterized by a diminished staining of the collagenous fibres when compared to the surrounding reticular dermis reflecting the deposition of still immature collagen. This study indicates that post-translational modifications resulting in, e. g. altered crosslink formation between type I collagen molecules may play an important role in determining the altered structural properties of the skin in different fibrotic diseases.

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The Effect of Long-Term UV Irradiation to the Basement Membrane in the Skin

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Chronic exposure to UV irradiation induces damage of the skin components, and this damage leads to alterations in the appearance of the skin, such as wrinkles. To investigate these alterations we studied the effect of long-term UV irradiation to the basement membrane in the skin using an animal model.

Hairless mice were irradiated with UV (UVB: 20 mJ per cm^2, UVA: 14 J per cm^2 three times per week for 10 wk). The changes of basement membrane components were evaluated with immunohistochemistry and RT-PCR.

In this study, we found that immunohistochemical staining for collagen type IV, VII and Perlecan were reduced. These results show UV irradiation causes damages to the basement membrane, and these damages would be an important factor of changes in the skin appearances.

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Severely, Damaged Amorphous Components of Skin Elastic Tissue in a Congenital Recessive Cutis Laxa

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The newborn, male child was the first child of healthy, unrelated parents. He was born from the fourth, this time uncomplicated pregnancy of the mother, with Apgar 8–9 and weighed 3450 g. Because of his bad general condition he was immediately taken to the intensive care unit. His severe dysmorphy, which showed the clinical picture of recessive congenital cutis laxa, was obvious at his birth. Another important abnormality was the reduction in the tone and the mass of skeletal muscles, nevertheless contractures developed in more joints. Serum CPK and LDH levels were pathologically increased.

Despite the intensive care the child died on the ninth day because of pulmonary atelectasy and pulmonary emphysema. Other morphological abnormalities discovered at autopsy were dilatatio ureteris dextri, foramen ovale apertum, ductus Botalli persistens. The ultrastructural examination of the skin revealed decreased and abnormal elastic fibers in the papillary and subpapillary dermis, with electrondens and severely damaged amorphous components.

This patient might represent a unique case of cutis laxa associated with severe myopathy. A De Barsy syndrome could be disclosed on the basis of histological and ultrastructural examination of the skeletal muscle.

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Smoking Downregulates Collagen Synthesis in Skin

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We have investigated whether smoking affects collagen synthesis in skin. The rate of skin collagen synthesis has been assessed by forming suction blisters on the skin of upper ventral forearm, and measuring concentrations of collagen propeptides PINP and PIIINP from the suction blister fluid, by using Radio Immuno Assay method. Collagen propeptides reflect the rate of ongoing collagen synthesis *in vivo*. The study population consists of 98 men, of whom 47 are current smokers and 51 are nonsmokers.

The mean PINP concentration was 95.6 μg per liter in smokers and 115.9 μg per liter in nonsmokers (Mann–Whitney $p = 0.09$). The mean PIIINP concentration was 34.9 μg per liter in smokers and 44.5 μg per liter in nonsmokers (Mann–Whitney $p = 0.05$). The results systemically show lower concentrations of collagen propeptides PINP and PIIINP in smokers compared with nonsmokers, in young smokers (<50 y) compared with young nonsmokers (mean PINP 102.4 *vs* 126.8 μg per liter, mean PIIINP 36.8 *vs* 52.0 μg per liter), and in older (≥50 y) smokers compared with older nonsmokers (mean PINP 90.0 *vs* 109.4 μg per liter, mean PIIINP 33.3 *vs* 40.1 μg per liter). Concentrations of PINP and PIIINP varied greatly in both smokers and nonsmokers, which decreases the statistical significance, but the tendency to lower propeptide levels was clear in smokers.

We conclude that skin collagen synthesis, measured by levels of collagen propetides PINP and PIIINP, is decreased in smokers compared with nonsmokers and this is evident also when the study population is divided into younger and older agegroups. Our findings could partly explain the mechanism of impaired wound healing previously reported in smokers.

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Topically Applied Ascorbic Acid Helps to Re-structure Chronically Photodamaged Human Skin

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Chronic sun exposure induces cumulative, long-term damage in the skin. Epidermal changes concern keratinocytes, melanocytes and Langerhans cells, and may ultimately lead to neoplastic degeneration, but the most frequent and obvious modifications observed in photoaging are related to the loss of elasticity and thinning of the dermis. Ascorbic acid (vitamin C) possesses an antiradical potential and is known to stimulate the collagen synthesis by fibroblasts. We have evaluated the influence of topically applied vitamin C on the structure and composition of chosen epidermal and dermal components in chronically photodamaged skin.

Sun-exposed skin areas in 10 female volunteers 50–60 y.o. were treated daily, for 6 mo, with 5% ascorbic acid in w/o emulsion or its placebo (assigned in a double-blind manner to the left or right forearm). After this period, skin biopsies were taken and processed for light and electron microscopy evaluation, i.e., overall architecture of the skin, distribution of elastic fibres, collagens, and vimentin expressing cells. Results obtained from the pairs of sites treated with vitamin C and placebo were decoded and analysed semiquantitatively. Sun exposure-related damage could be observed in all studied biopsies. Major differences related to the application of the active compound were noticed in the ultrastructure of superficial dermis. There were more normally structured elastic fibres and metabolically active fibroblasts compared to the placebo-treated side.

These observations suggest that a prolonged topical treatment with ascorbic acid, applied in an appropriate vehicle, may result in activation of a dermal synthesis of elastic fibres. Such an induction should be potentially beneficial for restoration of elasticity of the photodamaged skin.

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Interest of a 5% Vitamin C w/o Emulsion in the Treatment of Skin Aging: Effects on Skin Relief

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Vitamin C (L-ascorbic acid) is essential for life in humans. Since the discovery in the 1930s that vitamin C is the antiscorbutic factor much works have been undertaken to elucidate its mechanisms of action. In relation to dermatology are the role of vitamin C as reductant and in collagen synthesis. However, only a small amount of work deal with the interest of topical application of vitamin C in skin damages in which oxydative stress and collagen synthesis play a key role, such as skin aging.

The present study investigated, in 20 female volunteers (55–60 y) with photoaged skin (low neck), the effect of a 6-mo daily application of a 5% vitamin C w/o emulsion on changes in topographical, biophysical and mechanical properties of the skin. The study was conducted in double blind vs. placebo (cream devoid of vitamin C).

The method involved a negative replica of the skin area before and 3 and 6 mo after treatment with either the 5% vitamin C cream or the placebo. The relief of the replicas was measured using a three-dimensional laser microscope allowing a resolution of 1 μm. The 3D image obtained was then analysed using a new approach of skin surface morphology which allowed to classify skin furrows according to their depth, width and orientation. The furrow families can be classified in the three main categories, i.e., microrelief (0–10 μm depth), medium (10–20 μm) and deep furrows (>20 μm).

Results showed that there exists a highly significant increase in the density of skin micro relief as well as a decrease of the deep furrows on the side treated with the vitamin C cream. Clinical examination by the dermatologist as well as self assessment by the patients confirm the improvement of the skin relief (especially of the fine coarse wrinkles ($p < 0.001$)); thus confirming the relevance of the new approach of the measurement of skin surface morphology used in this study.

Our data clearly show that topically applied vitamin C can have a beneficial effect in the treatment of skin aging. Is the observed improvement of skin relief related to an activation of collagen synthesis in these patients, is a question we have undertaken to answer.

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Role of the p53-MDM2 Regulatory Feedback Loop in the Commitment to Human Squamous Differentiation

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The major *MDM2* oncogene product (p90) binds to the p53 tumour suppressor protein, inactivates its transcriptional activity and promotes its rapid degradation. In turn, the *MDM2* gene has been shown to be transcriptionally activated by p53, thus closing a regulatory feedback loop. We have previously reported the expression of MDM2 and p53 in Normal Human Primary Keratinocytes (NHPK), in the normal adult and *in vitro* reconstructed epidermis. To further explore the role of these proteins in human squamous differentiation we have analysed the spatio-temporal expression of MDM2/p53 in NHPK undergoing terminal differentiation. Taking benefit of the down regulation of β1 keratinocyte integrins during terminal differentiation, we have carried out suspension-induced terminal differentiation experiments. Alternatively, p53/MDM2 expression was studied in proliferative or terminally differentiating NHPK separated by their differential ability to adhere to collagen. The expression of p53 and MDM2 was also determined in epidermal cells derived from a human facial squamous carcinoma (SCC12B2) that undergo a delayed terminal differentiation in suspension. Dermal Human Primary Fibroblasts (HPF) were placed in suspension as well and analysed as a nonsquamous differentiating control. Temporal examination in both NHPK and SCC12B2 cells showed transient activation of MDM2 proteins prior to expression of terminal differentiation markers. Consistently, MDM2 up-regulation may account for progressive disappearance of p53 expression observed during the same period. However, whether *MDM2* transcription in squamous differentiation is p53 dependent cannot be ascertained. These changes occurred at the time during which NHPK lose their proliferative potential and are irreversibly committed to terminal differentiation. In contrast, no such regulation is found in the control HPF indicating a squamous differentiation specificity. Because of its critical role in the control of cell growth and proliferation, the expression of p53 must be tightly regulated and this implicates the negative feedback loop. Interestingly, our results suggest that MDM2 may be up-regulated prior to initiation of terminal differentiation to inactivate p53 when a tight control of the cell cycle is no longer required. The p53/MDM2 feedback loop might thus be required in controlling the correct relationship between cell-cycle arrest/proliferation and the commitment to terminal differentiation within the basal layer of human epidermis. Our results also indicate that the p53/MDM2 feedback loop may be active in the absence of any genotoxic stress, especially during a physiological process such as cell differentiation.

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Expression of β1 Integrin, Bcl-2 and Bax in Freshly Isolated Human Epidermal Cells. Implications for the Regulation of Human Epidermis Differentiation

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In the human epidermis, basal proliferating keratinocytes migrate upwards in the suprabasal compartment to become terminally differentiated. It has been previously shown that the expression of Bcl-2 and Bax, oncoproteins involved in apoptosis, is regulated during differentiation of keratinocytes *in vitro*. To further understand their regulation during differentiation *in vivo*, we have investigated their expression in freshly isolated human epidermal keratinocytes. The cells were prepared by trypsinization of abdominal skin biopsies and divided into two subpopulations based on their adhesion to type IV collagen. The total population, adherent and nonadherent subpopulations were immunostained with monoclonal antibodies against β1 integrin, implicated in the adhesion to basement membrane, Bcl-2 and Bax. We also double-immunostained for β1-Bcl-2 and β1-Bax. Cells were analyzed by flow cytometry and the populations of interest were sorted and further examined under the electron microscope. ‰[capsigma]0001[captau]β1 integrin expression was classified as $\beta1^{low}$, $\beta1^{medium}$ or $\beta1^{high}$; Bcl-2 as Bcl-2^{low} or Bcl-2^{high} and Bax as Bax^{low} or Bax^{high}. Adherent cells were mainly $\beta1^{high}$ (40%), Bcl-2^{high} (90%) and Bax^{low} (70%), whereas nonadherent cells were $\beta1^{low}$, Bcl-2^{low} and Bax^{high}. Double-immunostaining revealed strong correlations between β1 integrin, Bcl-2 and Bax expressions and four subpopulations were sorted: $\beta1^{high}$/Bcl-2^{high} and $\beta1^{high}$/Bax^{low} cells that had the morphological features of basal cells (plurilobate nuclei, rounded cells, melanosomes, sharp heterochromatin, dense cytoplasm with keratin filaments), and $\beta1^{low}$/Bcl-2^{low} and $\beta1^{low}$/Bax^{high} cells with features of suprabasal cells (big, rounded nuclei, elongated cells, no melanosomes, less heterochromatin, orientated keratin). Furthermore, among suprabasal cells, 50% are still Bcl-2^{high} and 70% are already Bax^{high} suggesting that Bcl-2 downregulation may occur later or to a less extent than Bax upregulation. Finally, Bcl-2 and Bax could be regulators of epidermal differentiation at both the basal and suprabasal levels.

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Induction of Keratinocyte Growth Factor and Hepatocyte Growth Factor/Scatter Factor in Human Primary Fibroblasts Cultured in the Presence of Keratinocytes and Interleukin 1β

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Keratinocyte growth factor (KGF) and hepatocyte growth factor/scatter factor (HGF/SF) are paracrine growth factors known to be involved in signaling between mesenchyme and epithelium. In order to investigate this interaction the production of KGF and HGF/SF in normal fibroblasts was measured after stimulation with the proinflammatory cytokines interleukin 1β (IL-1β), transforming growth factor β (TGFβ) and interleukin 6 (IL-6), and after coculture of fibroblasts with keratinocytes. Juxta-epithelial fibroblasts were isolated from three sites covered by epithelia with different differentiation patterns: skin (stratified orthokeratinized epithelium), buccal mucosa (stratified nonkeratinized epithelium), and gingival junctional mucosa (simple nonkeratinized epithelium). Following starvation for 3 d in culture medium containing 0.5% fetal calf serum, fibroblasts growing on plastic were incubated with fresh medium and stimulated with either cytokines or buccal/skin keratinocytes cultured on polycarbonate membranes. The KGF and HGF/SF protein levels in the culture supernatant were determined by ELISA after 24 h, 48 h, 72 h, and 96 h. The KGF and HGF/SF mRNA levels were estimated at 24 h using Northern blotting. We found that keratinocytes as well as Il-1β caused the fibroblasts to increase their production of KGF and HGF/SF protein 2–3-fold after 96 h. TGFβ inhibited the production of KGF and HGF/SF by 50% or more compared to control cultures, while IL-6 had no effect. Although the fibroblasts originated from sites with different epithelial differentiation patterns they showed the same response to stimulation with keratinocytes or cytokines, indicating that KGF and HGF/SF do not play a decisive role in determination of the biological differences between the three types of epithelium.

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Involvement of Epidermal CD40 in Keratinocyte Differentiation and Epidermal Remodeling

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Recent data support the idea that functional expression of CD40 on keratinocytes may be important for cutaneous inflammatory amplification. However little is known concerning the role of CD40 on normal epidermis. To address this problem, CD40 ligand-transfected L cells (CD40L) were used to analyze the effect of CD40 ligation on keratinocyte growth. Keratinocytes co-cultured with CD40L cells but not on CD32 transfected L cells, rapidly acquired morphological features of differentiated cells. This change consistently correlated with a significant thickness reduction of epidermal sheets (up to 50%), an effect that was mainly related to cellular flattening (evaluated by electron microscopy); and a transient CD40 low expression (measured by immunocytochemistry/image analysis). Moreover, anti-CD40 (M89 antibody) induced immunoreactive profilaggrin/filaggrin expression in multipassage keratinocytes under IFN-γ priming. Then, we assess the effect of IFN-γ on dermoepidermal sheets isolated from normal skin. As expected, a strong dosedependent immunoreaction to CD40 was localized chiefly on basal cells. In addition, basal cells from normal epidermis expressed largely the phenotype $CD40^+$/Ki-67^- whereas in areas of restricted proliferation were $CD40^-$/Ki-67^+. Hence, $CD40^+$ keratinocytes from freshly isolated epidermal cells ($CD29^+$) were mainly Ki-67^- while only 4.4% were $CD40^+$/Ki-67^+ double positives. Taken together, these findings support the concept that $CD40^+$ keratinocytes represent a poorly differentiated population, not actively engaged in the cell cycle, which under specific stimulation is committed towards growth and terminal differentiation affecting epidermal modeling.

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Molecular Cloning and Tissue Expression of the Murine Analogue to Human Stratum Corneum Chymotryptic Enzyme

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Human stratum corneum chymotryptic enzyme (SCCE) may play a central role in epidermal homeostasis. In order to facilitate further studies on SCCE we have looked for the corresponding murine enzyme. A cDNA obtained by RT-PCR from total RNA prepared from mouse tails was cloned, and the expression of the corresponding mRNA studied. The murine cDNA showed 77% homology to human *scce* cDNA. It had an open reading frame encoding a protein comprising 249 amino acids with 82% amino acid sequence homology to human SCCE including the conserved sequences of the catalytic triad of mammalian serine proteases. The murine protein was deduced to have a 21 amino acids long signal peptide and a 4 amino acids long propeptide ending with a tryptic cleavage site, followed by a sequence motif identical to the N-terminal amino acid sequence of native active human SCCE. As in human SCCE the P2 position of the propeptide was occupied by an acidic amino acid residue, and the position corresponding to the suggested bottom of the primary substrate specificity pouch occupied by an asparagine residue.

Analyses of mouse tissues by RT-PCR showed high expression in the skin, low expression in lung, kidney, brain, heart and spleen, and no expression in liver or skeletal muscle. *In situ* hybridisation of mouse skin showed expression in high suprabasal keratinocytes and in the luminal parts of hair follicles. Our results strongly suggest that we have cloned the murine analogue of human *scce* cDNA.

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Stratum CorneumTryptic Enzyme – A Missing Link in Desquamation?

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There is good evidence that stratum corneum chymotryptic enzyme (SCCE) may be important in desquamation. Other proteases, especially stratum corneum tryptic enzyme (SCTE), may also be involved. SCTE has been purified and its cDNA has been cloned. Expression analyses indicate that SCTE is as skin specific as SCCE. We have in this work produced and characterised antibodies specific for SCTE. We have also, by means of biochemical, immunochemical and immunohistochemical methods studied SCTE in normal human epidermis. Antibodies against bacterial recombinant SCTE were produced and purified by affinity chromatography. Immunohistochemistry with antibodies reacting with pro-SCTE showed a staining pattern similar to SCCE-specific antibodies, i.e., the expression was confined to cornifying epithelia where there is a need of desquamation-like processes. Extracts of tape strips with superficial human stratum corneum were found to contain intact pro-SCTE and SCCE as well as the catalytically active forms of both enzymes. SCTE and SCCE had maximal activity at pH 8, but both had considerable activity also at pH 5. 5. The results were compatible with a role of SCTE in desquamation. SCTE may, together with SCCE, take part in the degradation of intercellular cohesive structures in the stratum corneum, and/or function as an SCCE-activating enzyme. The presence in normal superficial stratum corneum of precursors as well as of active forms of SCCE and SCTE, and the activity of both enzymes over a broad range of pH-values, suggest a possible regulation of desquamation either by degree of activation of proteases involved or by the acidity of the stratum corneum intercellular space.

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Proelafin – a Cornified Envelope-Bound Protease Inhibitor is Converted to Soluble Elafin During Inflammation

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Proelafin is a keratinocyte-derived serine protease inhibitor with a transglutaminase substrate domain. It has been shown that proelafin is an integral part of the *cornified envelope* (CE) supposed to be one of the major compounds responsible for the proteolytic stability of the *stratum corneum*. Elafin is a soluble truncated fragment of proelafin that can be purified from psoriatic scales lacking the transglutaminase substrate motif. Elafin is a potent inhibitor of leukocyte-derived proteases and is hence involved in regulation of inflammation.

In this study we focus on the characterization of proelafin and on the mechanisms that lead to the release of soluble elafin during inflammation.

We expressed recombinant proelafin in the yeast, *Pichia pastoris*. The recombinant inhibitor was purified to homogenity using HPLC-techniques and the correct N-terminus was verified through N-terminal sequencing. We observed that purified proelafin is a strong inhibitor of elastase similar to elafin. By using guinea pig liver transglutaminase we saw the formation of larger aggregates demonstrating proelafin-proelafin cross-links *in vitro*. This is in accordance with proelafin cross-links found in the CE *in vivo*.

The N-terminus of proelafin contains several cleavage sites for chymotrypsin-like, elastase-like and trypsin-like activities. Interestingly we could isolate all predicted N-termini from psoriatic scales suggesting a biological principle: during inflammation bound proelafin is converted into soluble elafin by the action of proteases, thereby facilitating protection against cell detachment caused by leukocyte proteases.

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Trypsinogen IV in Human Epidermis

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There are many reasons to suggest that serine proteases are involved in physiological desquamation of human stratum corneum. We have now isolated a trypsin-like serine protease from extracts of human callus by specific affinity chromatography using immobilized benzamidine followed by reversed phase-HPLC. The purified protease was enzymatically active and substrate as well as inhibitor specifity classify this enzyme as a human trypsin, clearly distinct from uPA and human plasmin. In addition the isolated protease could be shown to be immunoreactive with an antihuman pancreatic trypsin antibody. This antitrypsin antibody was subsequently used for immunohistochemical analysis with formaldehyde-fixed and paraffin-embedded sections of normal human skin. The trypsin-specific immunoreactivity was confined to epidermal keratinocytes, with pronounced staining in the granular layer and the transition zone of stratum granulosum to stratum corneum. Using primers designed for the detection of human pancreatic trypsins, we performed RT-PCR with mRNA isolated from cultured primary keratinocytes. The obtained cDNA fragments showed complete nucleotide sequence homology with the gene encoding for trypsinogen IV. Further analysis revealed that both splicing forms of trypsinogen IV (the IV a-form and IV b-form) are expressed. The presence of trypsinogen IV mRNA could be also demonstrated in HaCaT-keratinocytes.

Our data demonstrate that beside urokinase-type-plasminogen activator and stratum corneum chymotryptic enzyme trypsinogen IV is the third serine protease that has been identified in human skin on mRNA and protein level.

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UVB-Induced Generation of 1α,25-Dihydroxyvitamin D_3 (Calcitriol) in Organotypic Cultures of Human Keratinocytes

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We recently demonstrated that epidermal keratinocytes *in vitro* can convert vitamin D_3 to biologically active $1\alpha,25(OH)_2D_3$ (calcitriol). Here we investigated (i) whether UVB-induced photolysis of 7-dehydrocholesterol (7-DHC) can result in the formation of calcitriol in cultured keratinocytes; and (ii) the wavelength of UVB involved. Human skin equivalents (HSE) were incubated in culture medium containing 1% (wt/vol) bovine serum albumin with 25 μM 7-DHC followed by irradiation with a HI-monochromator/Dermolum ($\lambda = 297 \pm 2.5$ nm; UV-doses: 7.5–60 mJ per cm^2). The UV-induced formation of calcitriol in HSE was time-and dose-dependent. A nearly linear increase of the calcitriol synthesis (≈0. 57 pmol per cm^2 HSE) was observed up to 16 h after irradiation. Unirradiated controls generated only minor amounts of calcitriol. The calcitriol formed was further identified as $1\alpha,25(OH)_2D_3$ by RP-HPLC and by GC-MS. UVB exposure at wavelengths between 285 nm and 320 nm and doses of 30 $mJ \times cm^{-2}$ resulted in similar curves and identical peaks of vitamin D_3 and calcitriol formation after 16 h of incubation. We found maxima of vitamin D_3 (106.2 pmol per cm^2 HSE) and calcitriol (≈0. 37 pmol per cm^2 HSE) generation 16 h after irradiation with UVB at 300 nm. Corresponding minima (42.4 pmol per cm^2 HSE and 0.07 pmol per cm^2 HSE) were observed at 292 nm. We were unable to detect vitamin D_3 or calcitriol generation at wavelengths ≥315 nm. Our data indicate a close relation between UVB-induced generation of vitamin D_3 and synthesis of calcitriol in HSE.

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Characterisation of Proteolytic Cleavage of Human Corneodesmosin During Epidermal Differentiation

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Human corneodesmosin (Cdsn) is a putative adhesion protein that is expressed in the upper spinous and granular keratinocytes. Secreted via lamellar bodies, it is associated to desmosomal cores just before transformation of desmosomes into corneodesmosomes. Synthesised as a 52–56 kDa protein, Cdsn is progressively proteolysed, the 52–56 kDa form being no longer detected at the skin surface where 36–30 kDa fragments are predominant. Further degradation of Cdsn was described as a prerequisite for corneocyte detachment.

The various steps in the proteolytic processing of Cdsn were characterised by immunohistochemistry and immunoblot analysis using three monoclonal antibodies specific for the central domain, and four affinity-purified antipeptide antibodies raised to several parts of the N-terminal (n = 2) and C-terminal (n = 2) domains of the protein.

In human epidermis, all of the reactive antibodies produced a cytoplasmic granular labelling of the granular layer. Some of them also stained the lower part of the stratum corneum. In sequential extracts of human epidermis, all of these antibodies immunodetected the 52–56 kDa form whereas only some of them detected 48–44 kDa fragments of Cdsn. In total extracts of superficial stratum corneum obtained by varnish-stripping, only two monoclonal antibodies and one antipeptide antibody detected the smaller fragments of the protein (30 kDa and less).

Our results indicate that the first step of proteolytic processing of Cdsn, probably before its incorporation into desmosomes, corresponds to the cleavage of its extremities. The N-terminal domain of the protein, formed by glycine loop-related structures, is then degraded. Finally, part of the C-terminal domain is cleaved. This multistep proteolytic cleavage of Cdsn would leave intact only the central domain of the protein that probably lacks adhesive properties. This may therefore allow desquamation.

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Ceramide 2(N-Acetyl Sphingosine) Induces G1 Arrest and Rb Dephosphorilation in Cultured Human Keratinocytes

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In the skin, the generation of intracellular ceramide may provide a link between an extracellular signal and the induction of apoptosis for the elimination of damaged cells. Previous studies have shown that cell permeant ceramides are involved in signal transduction, cell cycle regulation and apoptosis in different cell types. The aim of the present study was to investigate the intracellular apoptotic signal induced by permeant ceramides on cultured normal human keratinocytes (NHK). NHK from neonatal skin were cultured in serum free medium with or without Ceramide 2 (CER-2, *n*-acetyl-sphingosine) 20 μM, 40 μM. Cell cycle was investigated by FACS analysis, and western blot analysis of cyclin D1 and Rb dephosphorilation. Apoptosis was studied by TUNEL staining and western blot analysis of BCL-2 protein, p53 and p21. FACS analysis demonstrated a G1 arrest 48 h after ceramide addition, accompanied by down regulation of cyclin D1 and by Rb dephosphorilation. TUNEL staining showed the presence of CER-2-induced apoptosis after 48, 72 and 96 h of culture. Western blot analysis demonstrated that CER-2 induces a down-regulation of BCL-2 after 24 h up to 96 h, and an up regulation of p53 after 24 h. No modifications were noted in p21 levels. These results demonstrate that in NHK CER-2 cell permeant ceramide induces cell cycle arrest in G1, which precedes apoptosis.

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Upregulation and Activation of MMP-9 in HaCaT-*ras* Reconstructed Epidermis Model Compared with HaCaT and Normal Human Keratinocytes

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Matrix metalloproteinases (MMPs) play an important role in tissue regeneration, wound healing and tumor invasion. *In vivo* and *in vitro* studies reported on different tumors showed a correlation between 72 kDa (MMP-2), 92 kDa (MMP-9) type IV collagenase activities and neoplasia. In the present study, we compared the expression of MMPs in the spontaneously immortalized cell line, HaCaT and HaCaT-*ras* clones obtained after H-*ras* transfection, with normal human keratinocytes. These MMPs were studied in cells grown as monolayers in comparison with a reconstructed epidermis model using de-epidermized dermis (DED) seeded with these cell lines. The activities and expressions of MMPs were, respectively, detected by zymography and Western blotting. In this model, compared to normal human keratinocytes, the differentiation of epidermis is not completed as the stratum corneum is not detected, but a pluristratified epidermis is obtained with an intact basal membrane. In the reconstructed epidermis model, MMP-9 (92 kDa) is the major gelatinolytic activity secreted by HaCaT cell lines and normal human keratinocytes, whereas MMP-2 (72 kDa) activity was low and remained unchanged during all the cultures. In these experiments, MMP-9 active form (86 kDa) was detected earlier in HaCaT-*ras* reconstructed epidermis compared with HaCaT. From *in vitro* studies with the same cell lines, the MMP-9 active form was not detected, suggesting an important role of basal membrane, culture conditions or keratinocyte differentiation for its expression. The identification of main factors implicated in the activation of this gelatinolytic enzyme is under investigation. From these studies, MMP-9 could be an essential factor for tumor progression.

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Expression of β1 Integrin, Bcl-2 and Bax in Freshly Isolated Human Epidermal Cells. Implications for the Regulation of Human Epidermis Differentiation

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In the human epidermis, basal proliferating keratinocytes migrate upwards in the suprabasal compartment to become terminally differentiated. It has been previously shown that the expression of Bcl-2 and Bax, oncoproteins involved in apoptosis, is regulated during differentiation of keratinocytes *in vitro*. To further understand their regulation during differentiation *in vivo*, we have investigated their expression in freshly isolated human epidermal keratinocytes. The cells were prepared by trypsinization of abdominal skin biopsies and divided into two subpopulations based on their adhesion to type IV collagen. The total population, adherent and nonadherent subpopulations were immunostained with monoclonal antibodies against β1 integrin, implicated in the adhesion to basement membrane, Bcl-2 and Bax. We also double-immunostained for β1-Bcl-2 and β1-Bax. Cells were analyzed by flow cytometry and the populations of interest were sorted and further examined under the electron microscope. ‰[capsigma]0001[captau]β1 integrin expression was classified as $\beta1^{low}$, $\beta1^{medium}$ or $\beta1^{high}$, Bcl-2 as Bcl-2^{low} or Bcl-2^{high} and Bax as Bax^{low} or Bax^{high}. Adherent cells were mainly $\beta1^{high}$ (40%), Bcl-2^{high} (90%) and Bax^{low} (70%), whereas nonadherent cells were $\beta1^{low}$, Bcl-2^{low} and Bax^{high}. Double-immunostaining revealed strong correlations between β1 integrin, Bcl-2 and Bax expressions and four subpopulations were sorted: $\beta1^{high}$/Bcl-2^{high} and $\beta1^{high}$/Bax^{low} cells that had the morphological features of basal cells (plurilobate nuclei, rounded cells, melanosomes, sharp heterochromatin, dense cytoplasm with keratin filaments), and $\beta1^{low}$/Bcl-2^{low} and $\beta1^{low}$/Bax^{high} cells with features of suprabasal cells (big, rounded nuclei, elongated cells, no melanosomes, less heterochromatin, orientated keratin). Furthermore, among suprabasal cells, 50% are still Bcl-2^{high} and 70% are already Bax^{high} suggesting that Bcl-2 downregulation may occur later or to a less extent than Bax upregulation. Finally, Bcl-2 and Bax could be regulators of epidermal differentiation at both the basal and suprabasal levels.

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The Role of Protein Kinase C in HaCaT Keratinocytes

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Different protein kinase C (PKC) isozymes play crucial roles in the regulation of proliferation and differentiation of normal human epidermal keratinocyte (NHEK). In the present study, we have examined the possible involvement of the existing PKC isoforms in these processes in spontaneously immortalized HaCaT keratinocytes. The identification of different PKC isozymes and of differentiation markers (keratin 10, involucrin) was carried out by using Western(immuno) blotting, whereas the proliferation of the cells was monitored by bromo-deoxy-uridine cell proliferation ELISA kit. We have been able to detect several PKC isoforms (PKCα, β_{II}, γ, δ, ε, η, θ, ζ) in HaCaT cells which pattern differed from that of described in NHEK (PKCα, δ, ε, η, ζ). The expression levels of the different isozymes changed differentially during HaCaT cell proliferation and differentiation. For example, the PKCα and ζ showed marked expression in the cells regardless of their state of differentiation, whereas PKCε was practically not detectable in the confluent (hence differentiating) cultures. The PKC activator phorbol 12-myristate 13-acetate (PMA) inhibited HaCaT cell proliferation in a dose- and time-dependent manner. PMA also suppressed the expression of the early and late differentiation markers keratin 10 and involucrin, respectively, although with different dose–response relationships. These data suggest that HaCaT cells represent a suitable model for studying the role of PKC isozymes in keratinocyte biology, and that the proliferation and differentiation of HaCaT keratinocytes might be regulated differentially by different PKC isozymes.

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Characterization of Proteins Associated with Heat Shock Protein hsp27 in the Squamous Cell Carcinoma Cell Line A431

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Expression of hsp27 in human keratinocytes is associated with differentiation and its overexpression in a squamous cell carcinoma cell line inhibits tumorigenicity in nude mice. Hsp27 is a molecular chaperone and identification of hsp27-binding proteins might help to elucidate its functional role in keratinocyte biology. In the present study we used immunoprecipitation to identify proteins associated with hsp27 in the squamous cell carcinoma cell line A431. Cell extracts of hsp27-transfected and mock-transfected cells were incubated with a monoclonal antibody to hsp27 and adsorbed to an immunoaffinity column. The bound complexes were eluted and analyzed by immunoblotting. In accordance with earlier reports on the regulatory function of hsp27 on actin polymerization we found binding of hsp27 to actin. We were further interested in the interaction of hsp27 with other chaperones and found coprecipitation with the 70 kDa and the 90 kDa heat shock proteins but not with hsp60. Additionally, hsp27 was found to be associated with the mutated form of p53.

From these results we conclude: (i) Hsp27 is part of a complex system of molecular chaperones in A431. (ii) Investigation of the interaction of hsp27 and mutated p53 might shed new light on the development of epidermal tumors, since mutation of *p53* is an initial event in UV-induced carcinogenesis and since hsp27 – through its chaperone activity – might modify p53 function.

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Human Melanoma-Associated Chondroitin Sulphate Proteoglycan mRNA is Expressed in Normal Skin and Basal Cell Carcinomas

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Melanoma-associated chondroitin sulphate proteoglycan (MCSP) is a high molecular weight proteoglycan that is over-expressed in most melanoma cell lines. We have developed a monoclonal antibody LHM2 that reacts with MCSP and we have found that, in addition to strong staining of melanomas, LHM2 also strongly stains basal cell carcinomas, outer root sheath keratinocytes and a subpopulation of interfollicular keratinocytes but not squamous cell carcinomas of the skin. The MCSP gene has recently been cloned and was not found to be expressed in normal adult tissues. The aim of this study was to determine if the recently cloned MCSP gene is expressed in normal skin and if the gene is differentially expressed in BCCs.

RT-PCR identified MCSP transcripts in two of three melanoma cell lines, six of six normal skin samples, 10 of 10 BCCs and two of two cultured keratinocyte samples. Northern blot analysis using a gene specific probe showed strong expression of MCSP in BCCs but not normal skin. *In situ* hybridisation showed strong signal in BCC tumour islands but not in the adjacent normal skin. The expression pattern of MCSP and Gli1 in BCCs and normal skin is very similar. Although Gli1 is known to transcriptionally regulate Patched, there is little if any information on other transcriptional targets for this gene. We are therefore currently investigating the regulation of MCSP expression to determine if it is regulated by Gli1.

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Neutral and Acidic Sphingomyelinases are Both Induced by UVB Radiation in Normal Human Keratinocytes: Start Up Point for Ceramide Signaling

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The sphingomyelin pathway is an ubiquitous, evolutionary conserved signaling system which transduces extracellular signal into the cell. Cleavage of sphingomyelin by sphingomyelinase (SMase), evoked by different stimuli, causes the release of ceramide which can act as a second messenger for mediating apoptosis. Sphingomyelin can be hydrolysed by two types of SMases with neutral or acidic pH optimum, respectively. We have previously shown that treating normal human keratinocytes (NHK) with exogenous ceramides analogues can induce apoptosis in NHK. The aim of the present study was to investigate the role of neutral and acidic SMases and ceramide in the intracellular signaling generated after exposure (NHK) to UVB. NHK were cultivated with mitomycin-treated 3T3 cells in Dulbecco's modified Eagle's medium/Ham's F12 medium and, at preconfluency, were irradiated with a UVB dose of 100 mJ per cm^2. At different times after UVB irradiation cells were harvested for lipid extraction and for *in vitro* measurement of neutral and acidic SMase enzimatic activiy. Exposure to UVB radiation, resulted in rapid *in vivo* sphingomyelin hydrolysis and generation of ceramide as measured by TLC analysis. Ceramide accumulation peaked 15′ after UVB-irradiation. In vitro measurement of SMase activity from UVB-treated NHK extracts, using labeled sphingomyelin as substrate, showed an induction of both neutral and acidic SMase with slightly different kinetics. These data indicate that UVB can act on cellular membranes inducing sphingomyelin hydrolysis and ceramide production through both neutral and acidic SMAses.

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Magnetic-Activated Cell Sorting of Epidermal Langerhans Cells Yields Highly Purified Cells with Different Expression of Costimulatory Molecules

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Magnetic-activated cell sorting (MACS) is a recently developed immunomagnetic method for the isolation of cells which constitute only a minor subpopulation in tissues. We applied this method to establish a positive selection of human epidermal Langerhans cells due to their constitutive expression of the CD1a molecule. Epidermal cell suspensions were prepared from normal breast skin obtained from plastic surgery, and incubated with a microbead-coupled monoclonal antibody against CD1a. After immunomagnetic selection, the CD1a-positive cells were enriched from an initial frequency of 0.5–1.0% in the original sample to a purity of more than 90%. The cell suspension obtained showed an excellent ultrastructural morphology and a very high viability, with less than 5% dead cells as evidenced by propidium iodide staining. Immunophenotyping using triple color FACS analyses revealed a distinctive population of CD1a-positive cells with strong expression of CD11c, CD40, CD44, CD49f and CD50, and various expression of HLA-DR. There was no expression of the activation marker CD83, and most of the cells were also negative for the costimulatory molecules CD80 and CD86. However, we regularly observed a certain subpopulation of cells which expressed these costimulatory molecules. Taken together, the MACS system with CD1a represents a Very effective method for the careful isolation and enrichment of highly purified and viable epidermal Langerhans cells. The different expression of CD80 and CD86 suggests that the normal human epidermis harbours Langerhans cell populations with different functional properties.

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12(S)-Hydroxyeicosatetraenoic Acid Receptors may be Influenced by 2-Phenyl-methyl-1-Naphthol in Normal Human Keratinocytes

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2-phenylmethyl-1-naphthol (DuP 654) has been previously shown to be a lipoxygenase inhibitor and an anti-inflammatory drug in a murine skin inflammation model. Since 12-hydroxyeicosatetraenoic acid (12-HETE) is assumed to have a pathophysiological role in inflammatory skin diseases and epidermal cells posses high affinity binding sites for 12(S)-HETE, we studied the effect of DuP 654 on 12(S)-HETE binding to keratinocytes.

Radioligand binding assays were performed to determine 12(S)-HETE receptor characteristics on normal human keratinocytes.

DuP 654 antagonized 12(S)-HETE binding in a dose dependent manner with a K_i of 3.36 $\pm$ 0.23 nmol per liter. The antagonistic effect was reversible. On the other hand, after 1 and 24 h preincubation, the drug developed no significant inhibitory effect at the concentration between 10^{-10} and 10^{-5} mol per liter to specific 12(S)-HETE binding (B_{max} of 226 000 $\pm$ 27 000 receptors per cell) or to receptor affinity (K_d of 3.24 $\pm$ 0.28 nmol per liter).

Our results show that DuP 654, in addition to its 5-lipoxygenase inhibitory activity, exhibits 12-HETE receptor antagonist effect in normal human keratinocytes and therefore may be of benefit in skin diseases with elevated 12-HETE levels.

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Extracellular Calcium and TPA Differentially Regulate PKC Activation in Human Keratinocytes

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Keratinocyte terminal differentiation is abnormal in skin diseases such as psoriasis, emphasising the need to understand the factors regulating this process. $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor that regulates cell cycle progression. We have previously shown that upregulation of $p21^{WAF1}$ in response to increased extracellular calcium ($[Ca^{2+}]_o$) and 12-O-tetradecanoylphorbol-13-acetate (TPA) occurs through a protein kinase C (PKC)-dependent pathway in human keratinocytes (*Am J Pathol* 153:39–45, 1998). TPA induced a rapid and relatively transient increase in $p21^{WAF1}$ protein, whereas $p21^{WAF1}$ upregulation in response to increased $[Ca^{2+}]_o$ occurred in a slower and more sustained manner. The aim of this study was to investigate whether TPA and increased $[Ca^{2+}]_o$ differentially induce translocation of PKC isozymes and to compare the time-course of PKC isozyme translocation with the induction of $p21^{WAF1}$. TPA induced translocation of PKCa and PKCd from the cytosolic to the particulate fractions by 15 min and this was followed by PKC down-regulation by 18 h. In contrast, increased $[Ca^{2+}]_o$ induced a later translocation of PKCa and PKCd from the cytosolic to the particulate fractions which was not followed by PKC down-regulation (n = 4). These data show that TPA and increased $[Ca^{2+}]_o$ differentially activate PKC in human keratinocytes with the translocation of PKCa and PKCd following an earlier, but parallel, time-course to the induction of $p21^{WAF1}$ by these agents. Further studies are required to determine the role of PKC and $p21^{WAF1}$ in regulating keratinocyte cell cycle progression.

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Co-Expression and Functional Association of Cyclophilin A, FKBP12 and Calcineurin in Human Keratinocytes

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The immunosuppressant drugs cyclosporin A (CsA) and tacrolimus are effective treatments for psoriasis. In T-cells, CsA and tacrolimus bind to immunophilins, principally cyclophilin A and FKBP12, respectively, and inhibit calcineurin, a phosphatase required for T-cell activation. We have previously shown coexpression of cyclophilin A, FKBP12 and calcineurin in normal human skin and psoriasis. The aim of this study was to investigate whether immunophilins and calcineurin functionally interact in skin cells. Immunostaining in cultured human keratinocytes revealed a perinuclear (and to a lesser extent cytoplasmic) distribution for calcineurin and cyclophilin A whereas FKBP12 appeared predominantly nuclear. Treatment with TPA (50 nM) resulted in an altered intracellular distribution of both cyclophilin A and calcineurin to a more peripheral cytoplasmic pattern at 15 min–2 h, with the appearance of positive nuclear immunostaining at 18 h. Immunoprecipitation studies in human keratinocytes showed that CsA and tacrolimus promoted the association of calcineurin catalytic (A) and regulatory (B) subunits, providing evidence for the formation of a quaternary complex involving calcineurin A, calcineurin B, immunophilin and drug. These data support a functional interaction between immunophilins and calcineurin in cultured human keratinocytes and suggest that the therapeutic effects of CsA and tacrolimus in psoriasis may be mediated, in part, through T cell independent mechanisms.

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Effects of Hypoxia on the Murine Keratinocyte Plasminogen Activation System

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Cellular oxygen deprivation is a characteristic feature of wounded skin and malignant tumours, and has been suggested to occur in other pathologic cutaneous conditions. Activation of plasminogen, mediated by two distinct proteases, tissue type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), has also been demonstrated in many of these states. The effects of hypoxia on keratinocyte plasminogen activation is currently unknown and we were therefore interested to determine the effect of hypoxia on the components of the plasminogen system in the murine keratinocyte cell line, PAM212.

Cells were cultured under either normoxic or hypoxic conditions, and supernatants analysed for plasminogen activation activity by indirect colorimetric assay using the plasmin substrate S-2251. No significant differences in plasminogen activation were observed after 24 h, however, a marked increase was observed after 48 h hypoxia (n = 3). This increase was abolished by the addition of amiloride, indicating the presence of uPA, not tPA. Casein gel zymography confirmed induction of uPA after 48 h hypoxia with the presence of strong caseinolytic activity at 48 kDa. Northern blot analysis showed a rapid induction of PAI-1 mRNA after just 4 h of hypoxia continuing up to 24 h hypoxia, however, the mRNA level dropped below that of the normoxic after 48 h hypoxia. Surprisingly, uPA mRNA showed no significant changes over 48 h hypoxia. Immunoblot analysis of supernatants showed a gradual increase in PAI-1 protein expression over 48 h hypoxia. In contrast, no change was observed in PAI-2 protein expression after 48 h hypoxia.

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Stimulation of Glycosaminoglycan Synthesis in Keratinocytes by D Xylose

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The carbohydrates molecules, glycosaminoglycans (GAG), are involved in epidermal hydration and molecular water organization because of their water retention properties. Organized water has recently been shown to be of prime importance for the biological activities of the cells. Age related decrease in the content of GAG has been linked to changes in the biomecanical properties of skin during aging. We have investigated the capacity of pentose to stimulate GAG secretion by normal human keratinocytes by measuring ^{3}H-glucosamine uptake. The C 5 aldoses arabinose, ribose, ribulose, lyxose, xylose and xylulose were tested. Cells obtained from mammary plastic surgery were grown in K-SFM medium (Gibco) supplemented with bovine pituitary extract (50 µg per ml), EGF (5 ng per ml) and seeded in 96 well microculture plates at 25 000 keratinocytes per well. New serum free medium containing pentoses solubilized in water and 4 µCi 3H glucosamine was added after 24 h when the cells were confluent and culture continuated for 48 h. Supernatants were then collected and an equal volume of pronase (0.2 mg per ml) in PBS plus 0.02% sodium azide added for 12 h. The pronase was heat inactivated and labeled GAG were precipitated with a mixture of hyaluronic acid, dermatan sulfate, chondroitin sulfate 1/1/1 (8 mg per ml) and 40 µl cetylpyridinium chloride solution. The precipitate was collected, washed then disolved in methanol and radioactivity was counted. Pentose cytotoxicity was evaluated using the XTT test. Only xylose (1–10 mM) gave an dose dependent stimulation effect of GAG secretion (+55; +92; +113% for 1, 5 and 10 mM). Xylulose and mannose decreased GAG secretion over the same concentrations. The other aldoses tested had no effect. Furthermore, xylose had no effect on human fibroblast indicating a cell specificity pharmacological effect.

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Increased Level of Plasma Membrane Bound Matrix Metalloproteinase-9 by Ceramide Inhibits the Growth of Normal and Psoriatic Keratinocytes

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We recently reported that ceramide mediated inhibition of human keratinocyte growth coincided with overexpression of matrix metalloproteinase-9 (MMP-9); also batimastat (BB-94), as well as an MMP-9 blocking antibody could stimulate the proliferation of keratinocytes untreated or treated with neutral sphingomyelinase.We here demonstrate that, increasing the intracellular level of ceramide by Smase leads to accumulation of both pro-MMP-9 and active enzyme at the plasma membrane of human keratinocytes. That was particularly significant for cells from psoriatic patients which in untreated conditions exhibited low level of membrane associated MMP-9. Inhibition of psoriatic keratinocytes proliferation by Smase associated with high levels of cell bound MMP-9 could be partly reproduced with permeant truncated ceramides (C2 or C6 ceramide). In keeping with the known growth promoting activity of TIMP-1 for keratinocytes, we determined by Elisa the level of inhibitor produced by normal and psoriatic keratinocytes in absence or presence of Smase. Under our experimental conditions, psoriatic cells secreted a 2–3-fold increased of TIMP-1 but unexpectedly, Smase treatment enhanced inhibitor expression.

Overall, our data suggested that overproduction of active MMP-9 at the plasma membrane by ceramide could have beneficial value in psoriasis.

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Upregulation of Angiotensin Receptors in Artificially Wounded Human Keratinocytes

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Apart from its actions on the regulation of blood pressure and volume homeostasis, angiotensin II also influences proliferation and differentiation of diverse cell types. It plays a role in wound healing as well as tissue remodelling.

We recently showed the expression of angiotensin AT1 and AT2 receptors on human primary keratinocytes *in vitro*. In order to elucidate, whether the ratio of AT1 *versus* AT2 receptors changes in cutaneous wound healing, we used the common model of artificially wounding cultured human primary keratinocytes by razor blade scraping. Receptor expression was determined by semiquantitative RT-PCR 1, 3 and 12 h after scraping and compared to that on nonmanipulated cells.

AT1 receptors were upregulated 1 h after wounding and returned to baseline levels after 3 h. Upregulation of AT2 receptors was also detectable after 1 h, but reached maximum levels only after 3 h before returning to baseline levels after 12 h.

Our experiments indicate that both, AT1 and AT2 receptors are upregulated in wounded human keratinocytes. Since the expression of AT1 receptors peaks about 2 h earlier than the expression of AT2 receptors, we speculate, that initially after wounding the proliferative effect of angiotensin via the AT1 receptor dominates and promotes cell division, while later on the antiproliferative effect of angiotensin via the AT2 receptor more and more counteracts the AT1 receptor and thereby establishes an equilibrium in the cells' division rate.

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Protein Expression of Retinol Dehydrogenase in Psoriatic Skin and Basal Cell Carcinoma

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Retinoic acid (RA) affects the proliferation and differentiation of epidermal keratinocytes. These are processes that are disturbed in several dermatological disorders, including psoriasis and basal cell carcinoma. Like most other cell types, epidermal keratinocytes are able to produce RA from retinol. Several isoforms of the rate-limiting enzyme, retinol dehydrogenase (RDH), have been cloned from rat as well as mouse liver. Recently, a novel member of this family (RDH-4) was cloned from human liver, representing the first human microsomal enzyme specifically capable of oxidizing retinol. The presence of this enzyme in human epidermis has not previously been reported. Antisera raised against the N- and C-terminal fragments of RDH-4, respectively, were used for Western blotting of microsomes prepared from cultured human keratinocytes and for examination of the immunohistochemical localization of this enzyme in normal human skin as well as in psoriatic lesions and basal cell carcinomas.

In the microsomes from keratinocytes, the anti-N-terminal antiserum detected a 35-kDa RDH-4 protein. In normal and diseased epidermis, RDH-4 was found to be expressed with slightly increasing expression from basal to suprabasal layers. Several dermal structures such as the hair follicle epithelium, sweat glands and dendritic cells were also stained. When comparing healthy and diseased skin, no difference in staining intensity could be detected. In basal cell carcinomas, the tumour mass showed no expression unlike the surrounding stroma where RDH-4 positive cells were abundant.

The results reveal the presence of a retinol dehydrogenase in human skin, a candidate enzyme that might be responsible for the local conversion of retinol to RA.

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Immunocytochemical and Biochemical Identification of Angiotensin II in Human Skin and Keratinozytes in Culture

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Angiotensin II (ANG II), the active component of the renin angiotensin system, plays an important role in a wide range of biological processes including cardiovascular control, stimulation of DNA and RNA synthesis as well as cell proliferation. It has been shown in a variety of tissues such as heart, kidney, adrenal gland, kidney, testis, brain as well as in blood. The aim of this study was to determine the presence of ANG II in human skin.

ANG II was identified immunocytochemically in human skin from healthy volunteers, primary human keratinocytes and HaCat keratinozytes in culture. Likewise, ANG II-immunoreactive material was measured radioimmunologically in extracts of skin biopsies and cultured keratinocytes. The concentrations of ANG II were 82.96 ± 53.40 fmol per g wet weight (n = 6) for human skin biopsy samples, 8.41 ± 3.20 fmol per mg protein for primary human keratinocytes in culture (n = 3) and 19.17 ± 4.89 fmol per mg protein for HaCat keratinocytes (n = 4) (mean ± SEM). The ANG II-like material was characterized biochemically by HPLC on a reversed phase C_{18} column. Besides Ile^{5}-ANG II, ANG II metabolites such as ANG III, ANG $II_{3–8}$ hexapeptide and ANG $II_{4–8}$ pentapeptide were identified.

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Effect of Ultraviolet A1/B Radiations and Prostaglandin E2 on the Production of Endothelin-1 by Normal Human Keratinocytes

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In the skin, keratinocytes synthesize and secrete endothelin-1 (ET-1), a potent vasoconstrictor peptide which acts also as a paracrine growth factor for melanocytes, fibroblasts, endothelial cells and as an autocrine growth factor for keratinocytes. UVB irradiation increases ET-1 expression in cultured keratinocytes but the effects of UVA remain unknown. On the other hand, prostaglandin E2 (PGE2) is a vasodilator involved in UV-induced erythema and an autocrine mediator of keratinocyte proliferation but it is not known whether PGE2 itself could mediate its effects through a regulation of the expression of keratinocyte-derived ET-1. The aim of the study was to test the effects of UVA1 and the associations UVA1/B on the secretion of ET-1 by normal human keratinocytes cultured in serum-free medium, and to determine whether exogenously added PGE2 regulated ET-1 expression. Our results showed that UVA1 (365 nm) alone did not modify the level of ET-1 secretion and that the associations UVA1 + UVB or UVB + UVA1 down-regulated the overexpression of secreted ET-1 induced by UVB alone. Conversely, PGE2 whatever its concentration from 10^{-10} to 10^{-7} M did not significantly modify the expression of ET-1 at the mRNA or protein level. Neutral endopeptidase (NEP/CD10) known to degrade endothelins was not detected in the keratinocyte cultures and the addition of an anti-CD10 antibody did not modify the basal level of ET-1 secretion. Taken together, these results pointed out a differential regulation of ET-1 by UVB and UVA1 without any noticeable role for PGE2.

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Effects of All-*Trans*-Retinoic Acid on the Expression of Oncoprotein Bcl-2 by Cultured Normal Human Keratinocytes

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All-*trans*-retinoic acid (ATRA), the active metabolite of vitamin A, is an important regulator of the proliferation and differentiation of keratinocytes. ATRA inhibits terminal differentiation *in vitro*, blocking the expression of many markers of differentiation, including involucrin, tranglutaminase, and cornified envelope formation. Bcl-2 is an oncoprotein involved in the regulation of epidermal apoptosis and differentiation. We have investigated the effects of ATRA on Bcl-2 synthesis by cultured normal human keratinocytes by immunostaining and flow cytometric analysis. Human normal keratinocytes were grown in HK-SFM (Gibco) medium in 25 cm^2 flask for 72 h. New medium containing ATRA (10^{-6} or 10^{-7} M) was then added for 24 or 48 h. After trypsinization, cells were fixed in 2% wt/vol paraformaldehyde in PBS for 20 min at 4°C. Cells were then permeabilized with 70% vol/vol methanol for 40 min at 4°C and 4 μg monoclonal anti-Bcl-2 (Pharmingen) antibody was added for 1 h at 4°C. Cells were washed 2× and incubated for 1 h with fluorescent (FITC)-conjugated second antibody (Jackson Immunoresearch Laboratories): a $F(ab')_2$ fragment goat antihamster IgG antibody. The cells were washed 2× in PBS and analyzed by flow cytometry. ATRA increased the expression of the oncoprotein Bcl-2 in cells by 38% (10^{-7} M, 48 h, $p = 0.04$), 40% (10^{-6} M, 24 h, $p = 0.03$) and 51% (10^{-6} M, 48 h, $p = 0.01$). Bcl-2 synthesis was stimulated by ATRA in a time and dose-dependant manner. Thus, we suggest that ATRA could inhibit the terminal differentiation of keratinocytes by stimulating the expression of the antiapoptotic protein Bcl-2, a regulator of the epidermal homeostasis.

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Vitamin D_3 Analogues Exhibit Additional *In Vitro* Effects on Proliferation but not on Differentiation of UVB Treated Keratinocytes *In Vitro*

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The aim was to determine whether the addition of tacalcitol or calcitriol to UVB treated keratinocytes could produce an synergetic *in vitro* effect on cell proliferation and differentiation. The proliferation parameters were determined via the incorporation of 5 bromo-2'-deoxyuridine (BrdU) into the DNA and detected using a colorimetric immunoassay. A flow-cytometric analysis was performed to investigate the changes in expression of suprabasal keratin 10 and involucrin in keratinocytes. We found a dose-dependent decrease in DNA synthesis after exposure of keratinocytes (HaCaT cells and human keratinocytes) to UVB or vitamin D_3 analogues (tacalcitol and calcitriol). The combination of UVB (50, 100 or 200 mJ per cm^2) with tacalcitol [10^{-7}M] or calcitriol [10^{-7}M] offered a significant ($p < 0.01$) enhanced antiproliferative effect. After a single exposure to UVB we observed a dose-dependent induction of keratinocyte differentiation, indicated by keratin 10 expression, as well as induction of terminal differentiation, indicated by involucrin expression. There was no significant additional effect of vitamin D_3 analogues on cell differentiation. Our results suggest that clinical synergy of combining UVB with vitamin D_3 analogues like tacalcitol is rather due to an additional antiproliferative effect than due to increased keratinocyte differentiation.

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Treatment with 5-Fluorouracil Induces Caspase-3 Activation and Apoptotic DNA Degradation in HaCaT Cells

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5-Fluorouracil (5-FU) is frequently used in the therapy of various cancers as well as of actinic keratoses. It blocks DNA synthesis and causes deleterious structural changes of RNA which lead to inhibition of cell proliferation. In the present study, we investigated which apoptosis-associated processes are induced by these primary effects in immortalized keratinocytes. HaCaT cells were incubated with 5-FU at concentrations ranging from 10^{-9} to 10^{-3} M for up to 72 h. Concentrations higher than 10^{-6} M caused growth arrest and morphological changes typical for apoptosis. Caspase-3 activity was detected in lysates 24 h after treatment utilizing a chromogenic substrate. Further downstream apoptosis events such as nucleosome release into the cytoplasm as measured by ELISA and degradation of genomic DNA which was visualized on electrophoresis gels occurred in a concentration and time dependent manner. These effects could be blocked completely with a pan-caspase inhibitor. Our results suggest that caspase-dependent apoptosis is the main mechanism of 5-FU induced cell death in keratinocytes.

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Vascular Endothelial Growth Factor Secretion is Increased After Wounding of *In Vitro* Reconstructed Human Epidermis

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Vascular endothelial growth factor (VEGF) is produced by keratinocytes and has been implicated in the past as the major factor involved in wound healing associated angiogenesis. In the present study we investigated the production of VEGF by keratinocytes after wounding of *in vitro* reconstructed human epidermis (EpiDerm). For this purpose, these epidermal equivalents, consisting of differentiated epidermis without feeder layer of fibroblasts, were wounded by a 2-mm biopsy punch. Culture supernatants and epidermal lysates were analysed for VEGF after 4, 8 and 24 h. Wounding increased VEGF secretion 6.1, 3.1, and 3.3 fold after 4, 8 and 24 h, respectively, as compared to control, in four independent experiments. In contrast, no difference in the amount of stored VEGF could be detected in lysates of wounded and control epidermal equivalents. When transcriptional or translational activity was blocked by addition of actinomycin D or cycloheximide prior to wounding, secretion of VEGF was inhibited up to 70% indicating de novo synthesis rather than release of preformed VEGF after wounding. Our data demonstrate that VEGF secretion by KC in epidermal equivalents can be increased by wounding and that this regulation is independent of an interaction with mesenchymal cells.

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Heterogeneity of HaCaT Keratinocytes with Respect to UVA Induced H_2O_2: Role of Mitochondria and Degree of Confluence

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Exposure to ultraviolet radiation leads to induction of intracellular H_2O_2 in various cell types. In this study, we show that both basal and UVA induced H_2O_2 production in HaCaT keratinocytes is dependent on their degree of confluence. HaCaT keratinocytes were cultured for various time from 24 h till confluence. The intracellular levels of H_2O_2 were assayed using carboxy – H_2DCFDA, which is specifically oxidized by H_2O_2 to form fluorescent product carboxy-fluorescein. We show that the production of the fluorescent product strongly depends on the degree of confluence, being the highest in 24 h cultures with a decline during the increase of confluence. UVA irradiation resulted in a rapid and a uniform dose dependent increase in carboxy-fluorescein fluorescence in 24 h cultures. Interestingly, more confluent cultures are clearly heterogeneous with respect to UVA induction of H_2O_2 showing a presence of two main subpopulations with a lower and a higher sensitivity to UVA induced oxidative stress. Moreover, we show that the nonconfluent cultures are in general more susceptible to the UVA treatment as compared to confluent cells. The heterogeneity is related to the degree of confluence, most likely to cell-cell contact, but not to cell cycle phase. Inhibition of mitochondrial function resulted in a decrease in the number of high responding cells suggesting that mitochondrial activity is necessary for UVA induced H_2O_2 production in this subpopulation.

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Suprabasal (K1/K10+) Keratinocytes Produce NO and Kill Candida Albicans

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Human keratinocytes and HaCaT cells exert Candida albicans killing activity. Also, it has recently been reported that keratinocytes express inducable nitric oxide (iNOS). Therefore, we examined whether, similarly as with macrophages, the killing of Candida by human keratinocytes and HaCaT cells could be mediated by NO. Based on the characteristic suprabasal localization of Candida infection in the epidermis we also examined the difference in Candida killing and NO production between undifferentiated $K1/K10^-$ and differentiated $K1/K10^+$ keratinocytes separated by adherence to collagen. Keratinocytes were incubated with Candida cells at 37°C for 4 h. The killed Candida cells were counted after staining with methylene blue and NO was determined in the supernatant using a modified Griess method. Both keratinocytes and HaCaT cells killed Candida and produced NO. IL-8 and LPS + IL-8 pretreatment of the cells enhanced both the killing and the NO production by about 60%. L-NAME and W 1400 almost completely inhibited both the killing and the production of NO. $K1/K10^+$ differentiated cells were responsible for the killing as well as the NO production. Immunostaining of organotipic skin culture specimens treated with LPS + IL-8 showed mainly suprabasal expression of iNOS in the epidermis. This data indicate that killing of Candida is mediated by NO production of the differentiated, suprabasal keratinocytes in the skin. In the NO production iNOS plays a crucial role.

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P2Y_2 Receptor Activation Promotes Keratinocyte Growth and Decreases PTHrP Production

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When the skin is wounded, platelets release ATP, thus exposing the basal layer of the epidermis to raised concentrations of agonist at the G-protein coupled P2 receptors. Receptor activation results in increased intracellular calcium via changes in inositol phosphates, and triggers proliferation. Parathyroid hormone-related protein (PTHrP) release by keratinocytes (NHK) may be involved in regulation of proliferation. Therefore, we have investigated the action of ATP and UTP on growth and PTHrP production. Proliferation of NHK was assessed using a Coulter Counter. PTHrP production was analysed by immunoradiometric assay. After 24 h, 0.1, 1 and 10 μM ATP significantly increased the mean cell count (138 ± 6, 138 ± 6, and 147 ± 3×10^3 cells, respectively, n = 4) compared to controls (100 ± 4×10^3 cells, n = 8). PTHrP production was significantly reduced (198 ± 27.5, 236 ± 20.1, and 158 ± 6.9 fmol per 10^6 cells for 0.1, 1, and 10 μM ATP respectively) compared to controls (285 ± 38.8 fmol per 10^6). UTP had a similar effect. These results support the role of the P2Y_2 receptor in regulating NHK proliferation, with PTHrP as part of the autocrine signalling mechanism.

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Improved Basement Membrane Synthesis in Serum-Free Organotypic HaCaT-Cell Cultures by Retinoic Acid and TGF-β

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To facilitate the analysis of cell–cell and cell–matrix interactions, we had recently modified serum-free conditions for organotypic cocultures of normal keratinocytes or immortalised HaCaT-cells, both with dermal fibroblasts. Albeit herein differentiation markers appeared largely normal, basement membrane (BM) synthesis was inferior. This we tried to improve by specific supplements using HaCaT-cultures. Changes were analysed mainly by indirect immunofluorescence (IIF), but also by *in situ* hybridisation (ISH) and electron microscopy (EM). The addition of 1 ng per ml TGF-β or 10^{-8} M all-trans retinoic acid (RA), respectively, improved overall epithelial morphology but not the deposition of BM-components such as collagen IV, laminin-10, and nidogen. Rising RA to 10^{-7} or 10^{-6} M, although increasing synthesis of BM-material slightly, decreased markedly epithelial polarity showing irregular patterns, e.g., of keratins K1/K10 and of integrin α6β4. In contrast, combination of TGF-β and RA gave rise to clear structural improvements being optimal at 10^{-7} M RA. Furthermore, there was a nearly continuous linear decoration of the epithelial interface not only by BM-components but also by the hemidesmosomal constituents α6β4, BPAG-1 and −2, and HD1 as well as collagen VII. Preliminary ISH and EM data revealed corresponding improvements. Thus, our defined coculture model provides a basis for regulatory studies on normal and pathologically altered BM-formation.

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Influence of Advanced Glycation End Products on the Adhesion of Normal Human Keratinocytes

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The amino groups of proteins react with reducing sugars to form advanced glycation end products (AGEs). The skin content of AGEs increases with aging. We investigated the changes in the adhesion of normal human keratinocytes and their β1 integrin distribution caused by AGEs. Glycated type IV collagen was obtained by a 10-d incubation with ribose at 37°C, or with ribose plus tocopherol gentisate (100, 500 μg per ml). The general AGEs formation was monitored by 320/405 nm fluorescence on a microplate fluorometer (Fluostar, BMG). Cells were obtained by trypsinization of skin biopsies and plated out (37°C/1 h) on microplates or multiwell slide chambers coated with type IV collagen or glycated type IV collagen. Cells were incubated in medium alone (E-199 1% BSA), in medium plus 0.5 mM $MnCl_2$, or in medium plus the antioxydant tocophérol gentisate. Cell adhering to microplates were rinsed, fixed with methanol (–20°C/1 min) and their DNA measured (DAPI method). Cells adhering to slides were immunostained with antiβ1 integrin antibody. 31% fewer cells adhered to glycated collagen than to collagen (Anova, Newman-Keuls). $MnCl_2$ increased cell adhesion to glycated collagen by 33% and to normal collagen by 19%. β1 integrin immunostaining was found on the surface and on focal contacts of adherent cells in $MnCl_2$, on both normal and glycated collagen. Adherent cells in $MnCl_2$-free medium on glycated collagen were rounded, adhered poorly and had condensed areas of integrin immunostaining. Tocopherol gentisate increased the number of cells adhering to glycated collagen by 21% and the integrins in these cells had a normal distribution. Thus AGEs cause major changes in the interactions between keratinocytes and their extracellular matrix, and may influence epidermal homeostasis during aging.

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Subpopulations of a Murine Keratinocyte Tumor Cell Line Display Differential Sensitivity to Apoptotic Stimuli

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Tumor cell lines represent a convenient model system for the *in vitro* investigation of a multitude of biological systems. We have recently described the generation of murine keratinocyte cell lines derived from chemically induced sqamous cell carcinomas in K14 promotor driven bcl-2 transgenic and normal FVB/N mice. One of these cell lines, from a bcl-2 expressing transgenic mouse, was found to be defective in bcl-2 protein and mRNA expression, and was sensitive to cell-permeable ceramide, but not UVB induced apoptosis. In addition, this cell line had a mixed morphology of typical "cobblestone" keratinocytes as well as spindle shaped cells. In order to investigate whether the differential sensitivity to these two apoptotic stimuli could be ascribed to two separate cell populations, we have sub-cloned this cell line, and examined representative clones for cytokeratin 14 (CK14) expression and response to UVB and ceramide induced apoptosis. We find that whereas subclones from homogeneous parent cultures were uniformly cytokeratin 14 positive and displayed apoptotic responses as expected from their bcl-2 expression pattern, subclones from the heterogeneous parent line varied widely with respect to their degree of CK14 reactivity. Whether the CK14 negative cells represent a stable sub-population of dermal cells or dedifferentiated keratinocytes remains to be determined. These subclones also displayed different combinations of sensitivity/resistance to UVB and ceramide induced apoptosis, suggesting that multiple apoptosis regulatory pathways can exist within a tumor cell population. However there was no clear correlation between morphology, CK14 expression and apoptotic phenotype.

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Matrix Metalloproteinases and One of their Inhibitor in Squamous Cell Carcinoma and Zinc Modulation of their Expression

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Matrix metalloproteinases (MMPs), enzymes containing a Zinc atom, are involved in the development of some cutaneous carcinomas like Squamous Cell Carcinoma (SCC). We know that a few trace-elements, including Zinc, are able to modulate enzymes. The aim of this work was to study *in vivo* expression of MMPs and their inhibitor TIMP-1 in SCC among immunosuppressed and nonimmunosuppressed patients, and in addition to study *in vitro* the effect of Zn on MMPs and TIMP-1.

Modulation by Zinc was studied using normal keratinocytes monolayer culture with a concentration range between 1 and 20 μg per ml of Zinc metal (Labcatal, France) in culture medium. Furthermore, *in vivo* study of MMPs and TIMP-1 expression was led on biopsies from patients suffering from SCC at three different states: *in situ* carcinoma, undifferentiated and well differentiated states. Non-exposed normal skin was used as a control. Variations of expression were analysed by immunohistochemistry using the peroxydase technique. Cellular counting and a test with a marker of proliferation (Ki67) were added to this technique for *in vitro* study.

A dose-dependent modulation of MMPs and TIMP-1 by Zn with an increasing induction of their expression from 1 to 10 μg per ml (maximum of expression). This induction was correlated with a state of keratinocytes proliferation. A loss of cell/cell adhesion and an important mortality were observed for highest levels of Zn, probably related to a toxic effect at these doses. The three states of SCC among immunosuppressed and nonimmunosuppressed patients showed low levels of MMPs and TIMP-1 expression, whereas normal skin showed high levels.

In conclusion, Zinc could be used as a preventive in Squamous Cell Carcinoma for its capacity to increase MMPs expression and thus to induce loss of cell/cell contacts.

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Keratinocyte Adhesion Assays: A Comparison of Methods

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Cell adhesion has been well documented in immunological research, however, there is little literature available as to the problems that may be encountered when adapting these assays (predominantly developed for leucocytes) for use with keratinocytes. Until recently, keratinocyte adhesion has mainly been investigated by staining and manual counting or by other less sensitive colorimetric methods. With the increase in the number of fluorogenic probes available, their use as a sensitive alternative to radioactive labelling has been promoted in the literature.

This study was carried out to investigate the possibility of using a fluorescent probe (CFDA-SE) to assess the adhesion of keratinocytes and to compare this technique with the other techniques used to measure keratinocyte adhesion – the aim being to achieve a standardised assay for measuring keratinocyte adhesion.

We concluded that fluorescent probes might provide a greater sensitivity in measuring adhesion, however, they may be cytotoxic to keratinocytes. In addition the prelabeling may effect cellular functions such as adhesion and even proliferation and consequently probes must be chosen with care.

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Changes in Chromatin Structure During Differentiation of HaCaT Cells

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It is known that local changes in chromatin structure play an important role in the regulation of gene transcription, DNA replication and DNA repair. Here we studied global changes in chromatin structure during differentiation of HaCaT keratinocyte cell line. The cells were sampled from actively proliferating HaCaT cultures and from confluent cultures arrested in G1 phase of the cell cycle. Differentiation was induced by the treatment with calcium or calcium ionophore, ionomycin. DNA conformation was probed *in situ* employing a panel of fluorescent DNA probes: 7-aminoactinomycin (7-AAD), YO-PRO-1, and propidium iodide (PI). Fluorescence was measured by laser scanning cytometry, the principle of which resembles flow cytometry.

In confluent, growth-arrested cells nuclear DNA stainability with DNA probes decreased comparing with the actively proliferating cells. A further decrease in fluorescence took place during calcium- or calcium ionophore-induced differentiation. Experiments employing DNA-protein cross-linking with formaldehyde and histone removal with HCl revealed that the observed decrease in DNA stainability in the initial phases of differentiation was caused by an increase in DNA-histone. In contrast, in ionomycin-treated cells, histone removal did not significantly affect DNA stainability. Thus, chromatin conformation changes markedly during keratinocyte differentiation, a phenomenon which may be responsible for regulation of transcription and DNA replication in maturing cells.

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Characterization of Retinoid Metabolism Pathways in HaCaT Cells

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Vitamin A and its derivatives (retinoids) are essential for the regulation of a variety of life processes, such as vision, reproduction, embryonic development, and the regulation of cell growth and differentiation. There is also considerable evidence that retinoids are effective in the treatment of hyperproliferative, premalignant and inflammative skin diseases (e.g., psoriasis, Darier's disease, actinic keratoses and acne). Although profound progress has been achieved with regard to the action of retinoids on the molecular level, the metabolic pathways of retinoids in normal and malignant tissues are largely unclear. Since stable intracellular retinoid-levels appear to be essential for optimal retinoid receptor function, irregularities in the pathway of retinoids may have profound effects here. The cascade of retinoid activation is driven by enzymes of the alcohol dehydrogenase family (ADH), short chain dehydrogenases (SDH), aldehyd dehydrogenases (RalDH), and several enzymes of the P450 isoenzyme superfamily (CYPs). The inactivation of *all-trans*-retinoic acid has been shown to be mediated by the novel CYP26. Using the human epidermal HaCaT cell line, we investigated the pathways of retinoid metabolization and isomerization on the biochemical level by reverse phase-high performance liquid chromatography (RP-HPLC), and on the molecular level by rt-PCR to estimate the expression-levels of several retinoid-related RNAs, such as RolDH, RalDH, cellular retinoic acid binding protein-II (CRABP-II), and CYP26 (internal control: beta actin). HaCaT cells were incubated with either 10^{-5} M *all-trans*-retinol (ROL), *all-trans*-retinal (RAL), *all-trans*-retinoic acid (atRA), *9-cis*-retinoic acid (9cRA), or *13-cis*-retinoic acid (13cRA). Specimens for RP-HPLC and rt-PCR were obtained 0, 24, 48, 72, 96, and 120 h of incubation, respectively. As observed by semiquantitative rt-PCR and RP-HPLC analysis, HaCaT cells exhibit RolDH, RalDH, and CYP26 mRNA activity. After ROL-stimulation, no RAL and RA-isomers were generated. After RAL-induction, HaCaT cells formed high levels of ROL, RA-isomers, and RA-metabolites. After stimulation with either RA-isomer, very high levels of CYP26 activity were detectable on the biochemical (RP-HPLC) and on the molecular level (rt-PCR), while the expression of ROLDH and RALDH was suppressed. We conclude that HaCaT cells are able to interconvert RAL exhibiting inducible RalDH and CYP26 activity, while RolDH has a weak basal expression without inducibility. These results suggest that, in contrast to normal human epidermal keratinocytes, the pathways of retinoid metabolism and the activity and inducibility of the enzymes involved here seem to be severely altered in the HaCaT cell line. For comparison, results of the concerned enzymes in the retinoid metabolism in normal human keratinocytes will also be presented.

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Response of Nonlesional Atopic and Nonatopic Skin to Changes in Thermo-Hygric Atmospheric Conditions

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On the background of well-known disturbances of the barrier function and vasoreactivity in atopic skin the aim of the present study was to investigate adaptative temperature regulation reactions of atopic skin in comparison to normal skin. In a climate simulation chamber atopic subjects (n = 12) and nonatopic controls (n = 10) were exposed to four climatic conditions (const. air temp. 22°C), differing in terms of relative humidity (RH) of the air, wind velocity (WV) and physiological equivalent temperature (PET): (50% RH, 0 m/s WV [PET: 22.5°C] (50% RH, 2 m/s WV [PET: 18.1°C] (80% RH, 0 m/s WV [PET: 22.9°C] (80% RH, 2 m/s WV [PET: 18.4°C]. Skin measurements were performed on unaffected skin of the volar forearms. Generally, the quantity of transepidermal water loss (TEWL) and water content (WC) of the stratum corneum were mainly influenced by RH, whereas cutaneous blood flow (BF) and skin temperature (ST) varied with alterations in WV resp. PET. Atopic skin tended to show higher baseline values (7.3 *vs* 5.7 g/m^2 h) and a stronger humidity-induced decrease (45% *vs* 25%) of TEWL, resp. lower basic values (67.5 *vs* 74.1 AU) and a stronger increase (32% *vs* 25%) of WC with rising RH, as well as to stronger changes in BF (+40/–34% *vs* +37/–3%) in response to alterations in PET, but the differences to nonatopic skin were not significant. The results show that noneczematous atopic skin does not generally react differently in adaptational evaporative and vasal skin responses to thermo-hygric atmospheric changes; however, there is a trend to increased responses to alterations in RH and PET, which might become relevant under certain conditions in the otherwise functionally disturbed lesional atopic skin.

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Relationship Between Detergent Induced Inflammation and its Penetration Assessed by Microdialysis Technique

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To understand the inter individual variability in irritation, induced by sodium lauryl sulfate (SLS) and other surfactants, knowledge of the relationship between the penetration of SLS through the epidermal barrier and the induced irritation is necessary. To obtain relevant pharmacokinetic data, SLS penetration was studied in human volunteers by microdialysis (MD). The degree of irritation was assessed by clinical score, and increase of total protein content in the dialysate as an endogenous marker for irritation. Barrier function was assessed by TEWL. After 12 h of pre-exposure to 5% aqueous SLS (50 ml), we inserted two linear probes (MW cut off: 3000 kDa) into the dermis of the volar forearm of healthy human volunteers (n = 8). The probes were perfused with 5% glucose solution (flowrate 1 ml per min). Samples were collected in 1 h intervals for the duration of 3 h. The dialysate was analyzed for SLS (Mobile Phase Ion Chromatography) and total protein content (photometrically, using Coomassie blue dye). Clinical assessment and TEWL were performed before and after the experimental procedure. A clear correlation was found between total protein content and clinical score. This indicates that the increase of total protein content in the dialysate is a good endogenous marker for dermal inflammation. Additional a correlation was found for irritation (TEWL, clinical score) and the increase of the amount of SLS penetrated into the dermis. The results indicate that possible reasons for the correlation between irritation and the amount of SLS in the dermis are: (a) The subject related variations in barrier function. This leads directly to different amounts of SLS penetrating to the dermis and (b) The different susceptibility of individuals to SLS, which leads to differences in inflammatory response. The inflammation might facilitate the penetration, due to an influx of oedema and widening of the intercellular spaces and therefore, indirectly, increase the amount of SLS detected in the dermis.

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Effects of Two Specific Lipidic Emulsions on the Neosynthesis of Epidermal Lipids on Human Skin Explants in Culture

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Lipidic furans and polyhydric fatty alcohol from Avocado Unsaponifiables and Sunflower oil Oleo Distillate obtained by molecular distillation (80% Essential Fatty Acids; 5% phytosterols), have chemical structure which could theoretically interacts with lipid metabolism. The effect on epidermal lipids of thus O/W emulsions containing 2% of Avocado Unsaponifiables or Oleo Distillate, *versus* excipient, was investigated by an *in vitro* assay using human full thickness skin explants (from abdominal plastic residues). The skin explants were incubated for 18 h, under non submerged conditions, in presence of ^{14}C-acetate used as radiolabelled precursor of lipids. The neosynthesis of di and triglycerides, cholesterol (-sulfate), cerebrosides, ceramides 1 and 2 was studied by the measurement of the incorporation of the ^{14}C-acetate in the newly synthesised lipids extracted and separated by thin layer chromatography. The lipid neosynthesis was analysed according to standard lipids. Epidermal Growth Factor (10 ng per ml) was used as positive reference. The results were:

Epidermal Growth Factor: increase in the neosynthesis of ceramide 1, ceramide 2 and cholesterol sulfate.

Excipient: down regulation of the neosynthesis of di-triglyceride, no effect on other lipids

Avocado Unsaponifiables: high increase in the neosynthesis of cholesterol sulfate, cerebrosides, ceramide 1, ceramide 2 and cholesterol

Oleo Distillate: high increase in the neosynthesis of ceramide 1, ceramide 2 and cholesterol.

The results demonstrated that Avocado Unsaponifiables and Oleo Distillate topically applied on human skin could modulate epidermal lipid pattern *in vitro*. The increase of ceramides and cholesterol suggested an implication in the regulation of desquamation and in the permeability barrier homeostasis.

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Regional Heterogeneity in Hydrophobicity of Cornified Envelopes from Human Stratum Corneum

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Cornified envelope (CE) is a rigid and insoluble structure of corneocyte in the stratum corneum, which is assembled by crosslinking of several precursor proteins by transglutaminases. CEs provide a foundation of barrier function by covalent attachment of w-hydroxyceramides to the outer surface of CE components, followed by organized lamelar layer of intercellular lipids. It has been reported that morphologically irregular fragile CEs are found in the deep layer of the stratum corneum or certain disorders, such as psoriasis, whereas most of CEs from healthy subjects are rigid and polygonal. Here, we report regional difference of properties of CEs from healthy subjects, especially regarding with hydrophobicity and barrier function of the skin.

Stratum corneum of healthy volunteers were collected by tape stripping, and CEs were prepared by extensive boiling and washing in buffer containing 2% SDS and 20 mM dithiothreitol. Hydrophobicity of CEs were examined by staining with Nile red dye. Involucrin in the CEs was detected by fluorescence immunohistochemistry.

CEs from the upper arm were relatively homogeneous in hydrophobicity as well as morphology with larger shape. CEs from the face were strikingly heterogeneous and consisted of rigid CEs and fragile CEs. Rigid CEs were Nile red positive and weakly stained by anti-involucrin. Most of them exhibited envelope-like morphology with contour staining as observed by confocal laser-scanning microscopy, whereas some of them were completely stuffed with hydrophobic materials. In contrast, fragile CEs were Nile red negative but strongly stained with anti-involucrin in mutually exclusive manner. These results suggest that properties of CEs were varied depending upon region even in healthy subjects, and may reflect relationship between appearance of immature CEs and barrier impairment.

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Effects of Vehicle on "*In Vitro*" Percutaneous Penetration of Benzophenone-3

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Benzophenone-3 is usually employed to protect against detrimental effects of UV light on human skin. Ultraviolet-filters need to be maintained on the skin to obtain the expected protection. These products were applied repeatedly to exposed skin over a large surface approximately 1.8 m^2 for 70-kg adult. In order to ensure adequate protection, the percutaneous penetration and drug tissue concentration must be examined and quantified. Percutaneous absorption was assessed *in vitro* using a diffusion cell skin absorption model. The static diffusion Franz cell consists of donor and receptor chambers between which the skin is positioned. Excised pigskin is well accepted to estimate the *in vitro* dermal penetration. For the series of experiment reported, pig ears were used. Five vehicles were employed to deliver benzophenone-3 to the skin surface, three solvents (coconut oil, capric-caprilic triglyceride and propylene glycol) and two emulsions (oil/water and water/oil). Under these conditions the quantity of product applied to the surface was 2 mg cm^{-2}, which corresponds to the FDA recommended test dose for measurement of the UV light protection factor. The application times were 1, 2, 4 and 8 h. The results demonstrate a vehicle effect on the skin penetration of benzophenone-3. The two oily solvents restrain the movement of this UV-filter through the skin and whereas propylene glycol and emulsions allow a greater penetration. Benzophenone-3 was always detected in the skin at concentration with time, and the levels are more important with propylene glycol and emulsions.

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Sodium Lauryl Sulfate Causes IL-1α Release from Normal Human Keratinocytes: Dose Response and Time Course Curves

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Interleukin 1α (IL-1α) is involved in the biological response to irritants as one of the most important inflammatory mediators. The aim of the present study was to evaluate IL-1α production after exposure to Sodium Lauryl Sulfate (SLS) from cultured human keratinocytes, representing a model which enables to asses the *in vitro* effects of SLS on epidermal cells independently from the skin immune system and barrier alterations.

Normal human keratinocytes from plastic surgery were grown in serum free medium. At subconfluency cells were treated with SLS doses ranging from 0.00001 to 0.005%. After one hour exposure the medium was changed. At different time points the supernatant was collected for ELISA, and cells were harvested for Western blot analysis of pro-IL-1α and IL-1α.

Extracellular secretion of IL-1α from keratinocytes was increased in a dose dependent manner following SLS treatment. The release of IL-1α starts at 30 min after exposure to SLS (DL_{50}) reaching a maximum at 3 h.

Western blot analysis showed a down regulation of pro IL-1α levels at 1 h, while IL-1α levels remained unchanged.

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Epidermal Physiology at Epicutaneous Patch Testing for Ni-Allergy Assessed by PIXE

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The skin penetration *in situ* profiles of common metals known for their capacity to induce contact allergic reactions, e.g., Cr, Ni, Cd, and Hg, has not been demonstrated as yet. Using proton induced X-ray emission analysis (PIXE) which is sensitive to trace element levels, i.e., < 1 μg per g, we have been able to demonstrate that in patch tested skin, Ni accumulates in the stratum corneum and is not found in the bulk of the viable epidermis. This result suggests that a specific binding of Ni occurs in the stratum corneum and that the amount of Ni able to elicit a contact allergic reaction is indeed extremely low.

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Assessment of Solvents Effects on Human Skin Microtopography

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A number of solvents are manipulated daily in laboratories for biological and industrial usages. The aim of this work was to assess human skin surface alterations after some solvents contact.

After establishing an accurate method for limiting skin areas to study, replicas of silicone rubber (Silflo) were performed on the volar forearm surface of a young woman (20-y-old), before and after deposit of current laboratory solvents (water, ethanol, acetone, propylene glycol).

Skin surface modifications were detected by Scanning Electron Microscopy and visualised as well as assessed by Confocal Laser Scanning Microscopy. The results of this work demonstrated that the maximum of alterations were provoked by acetone and propylene glycol solvents. There were weak effects with water and ethanol.

However, it appears that the contact times (30, 45 min), which were adopted in our experiments, as quoted in the literature, should be increased to assess better the alterations of the skin surface after solvents contact.

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Effects of Tensides on Normal Human Keratinocytes: A Decrease in the Toxic Potential When Used in Association

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Sodium lauryl sulfate is an anionic tenside that is widely utilized as a model for studying acute and cumulative irritation. When used in culture, SLS induces an inhibition of DNA synthesis and a decrease in cellular viability. It has been previously reported that the barrier damage caused by SLS *in vivo* is lower when SLS is applied to the skin in combination with other tensides.

The aim of our study was to evaluate if the cytotoxic effect of SLS is reduced by the association with different tensides also *in vitro*.

Normal human keratinocytes from plastic surgery were grown in serum free medium. At subconfluency cells were treated with SLS at a dose corresponding to the DL50 in combination with Tween 20, Tween 80, Tegobetaine F50 at the minimum toxic dose.

Following tenside treatment, the culture medium was changed and after 24 h the cells were collected for thymidine incorporation, MTT assay and NR uptake.

The cytotoxic effect on normal human keratinocytes as evaluated by thymidine incorporation, MTT assay and NR uptake was significantly decreased by the combination with all the tested tensides.

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ErbB-2 Overexpression but No Activation of β-Catenin Gene in Extramammary Paget's Disease

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Our previous study in extramammary Paget's disease (EMPD) (*Br J Cancer* 76:904–908, 1997) showed neither p53 mutations nor allelic loss at selected loci implicated in other cancers, suggesting the different pathogenesis of this particular form of skin cancer from other common epithelial malignancies. To examine further the genetic defects in EMPD, we carried out molecular genetic analyses in 31 tumor samples obtained from 27 cases of EMPD without underlying malignancies. Immunohistochemistry using CB-11 monoclonal antibody revealed either membrane or cytoplasmic erbB-2 oncoprotein overexpression in none of the 13 *in situ* primary tumors, but in one recurrent *in situ* tumor, 10 of 13 invasive primary tumors and two of four lymph node metastases. Sensitive dual color fluorescence *in situ* hybridization analysis using probes for *erbB-2* gene locus and chromosome 17 pericentromere, however, revealed different *erbB-2* gene status in the erbB-2 overexpressing tumors. One recurrent *in situ* tumor and one lymph node metastasis showed definite gene amplification characterized by multiple scattered signals or a few large clustered *erbB-2* signals, whereas four tumors with predominantly cytoplasmic erbB-2 overexpression were thought to have low-grade gene amplification. The remaining six tumors overexpressing erbB-2 showed no increase of *erbB-2* copy numbers. The additional studies demonstrated no evidence of abnormal activation of the β-catenin gene, a critical mediator of *Wnt* signaling pathway, in any tumor by immunohistochemical staining and by direct sequencing and RT-PCR analyses. Frequent overexpression of *erbB-2* by either gene amplification or possible transcriptional activation in invasive primary tumors and metastases suggests an important role of this oncogene activation in the progression of EMPD.

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Implication of p16 in Cutaneous Squamous Cell Carcinoma of Transplanted Patients

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The aim of our study was to compare genomic abnormalities between different populations of squamous cell carcinoma (SCC) using two techniques: comparative genomic hybridization (CGH) and loss of heterozigosity (LOH).

Three groups of SCC were constituted: non transplanted patients/transplanted patients/patients with recurrence of carcinoma or metastatics adenopathies.

Our CGH results combined with those from others allowed us to determine three interesting loci: 9p21/9p22.3/17p13. Potentially carcinogenic genes are known to be localized within these loci: p16/XPA,PTCH,MSSE/p53, respectively. Thirteen microsatellites within these three loci were then studied with an automatic sequencer. Seventeen biopsies have been studied including six, seven and four patients for the three groups, respectively. The LOH was more important for transplanted patients than for non transplanted (40/91 *vs* 35/130; $p < 0.05$). For 15 of 17 (88%) of patients we found LOH within the 17p13 locus, confirming that p53 defect is an early event in the development of SCC. The alteration of 9q22.3 locus (XPA,PTCH,MSSE) was found for 12 of 17 patients (88%) without significant different results between the three groups. For the 9p21 locus, a LOH was found for 11 of 17 patients (65%). P16 is thus likely to be implicated in SCC development as in melanoma. P16 defect was found in all the transplanted patients but rarely observed in non transplanted (four of 10 *vs* seven of seven; $p < 0.05$). Our data show significant differences between transplanted and non transplanted patients leading to the hypothesis that carcinogenic mechanism of SCC may be different depending upon patient diasthesis.

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Differential Gene-Expression in Basal Cell Carcinoma: Analysis by Differential Display PCR and cDNA Expression Arrays

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Basal cell carcinoma (BCC) is the most common cancer in humans. According to epidemiologic studies cumulative exposure to sunlight especially in the first two decades of the individual live plays a major role in the carcinogenesis of BCC. UVB-mediated mutations of DNA could lead to unphysiological expression of cellular genes and altered proteins. Thus, the phenotype of the malignant keratinocyte should be associated with an altered expression profile compared to the normal counterpart of the tumor cell. To detect such differentially expressed genes we used differential display PCR (DD-PCR) and differential hybridization of commercial cDNA expression arrays. For the DD-PCR analysis 10 BCC and three normal skin samples were displayed in parallel. In addition to six known genes also 15 new, BCC-associated sequences could be identified. For differential hybridization two identical cDNA expression arrays were probed with labeled cDNA-pools of either eight different BCC-specimens or three different normal skin-specimens. By comparing the signal intensity on the two arrays five repressed and eight induced genes were identified. Two ribosomal-proteins (40S RP-S19, 60S RP-L6) showed the strongest repression in the group of repressed genes. Among the induced genes the monocyte-chemotactic-protein 1 showed the strongest induction. In summary, both approaches allow the detection of differentially expressed genes possibly involved in the pathophysiology of BCC. The DD-PCR turned out to be a useful method to detect known as well as new genes in a multitude of samples at the same time. The cDNA arrays are restricted to prearranged known sequences but allow a faster identification of differentially expressed candidates.

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Association of Quinone Oxidoreductase Genotypes with Basal Cell Carcinomas

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We have reported associations between increased numbers of basal cell carcinomas (BCC) and the glutathione S-transferase GSTM1 B and GSTT1 null and, cytochrome P450 CYP2D6 EM genotypes. We speculate that other loci determining cellular response to oxidative stress such as NAD(H): quinone oxidoreductase (NQO1) are potential candidates for outcome in BCC. Accordingly, we assessed the association between NQO1 null and BCC numbers. Our main aim, was to rank the relative influence of NQO1 null and other genotypes such as GSTM1 B, GSTT1 null and CYP2D6 EM. We found 3.2% of cases were NQO1 null allele homozygotes. The mean number of BCC in *NQO1*0* homozygotes was greater than in wild type allele homozygotes and heterozygotes ($p = 0.06$). We examined the data for associations between BCC numbers and interactions between NQO1 null and putatively risk genotypes at the other loci. The four cases with NQO1 null and GSTT1 null suffered more BCC ($p = 0.04$). The relative influence of NQO1 null on BCC numbers was studied in a model that included: i. 241 of the 403 patients in whom GSTM1 B, GSTT1 null and CYP2D6 EM genotype data were available and ii. 101 of the 403 patients, in whom these genotypes as well as data on GSTM3, CYP1 A1 and melanocyte stimulating hormone receptor (MC1R) genotypes were available. NQO1 null ($p = 0.001$) and MC1*R* asp294 hi ($p = 0.03$) were linked with BCC numbers and the association with CYP2D6 EM approached significance ($p = 0.07$). In a stepwise Poisson regression model only these three genotypes were significantly associated with BCC numbers, with NQO1 null being the most powerful predictor.

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Genetic Changes in Sweat Gland Carcinomas

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To examine for the molecular pathogenesis of skin appendageal malignancies, we performed molecular genetic analyses in a mixed group of 21 sweat gland carcinomas. Loss of heterozygosity (LOH) was detected in four tumors. LOH was mostly confined to the chromosome arm 17p, suggesting that the inactivation of p53 or another target tumor suppressor gene on this chromosome arm plays an critical role in the pathogenesis of sweat gland carcinomas. None of the remaining 17 tumors showed LOH at any loci. Nuclear accumulation of p53 protein was observed in three tumors, all of which also showed LOH of 17p. Direct sequencing of the p53 gene in four tumors showing allelic loss of 17p revealed a CTC to CGC mutation at codon 176 that would alter a cysteine to arginine in an eccrine gland adenocarcinoma. The other three tumors, however, had wild-type p53 genes. In contrast to cutaneous squamous cell carcinomas (SCC), the inactivation of p53 seems to be a late event, as we observed a clear transition from benign poroma to porocarcinoma that was associated with p53 protein stabilization and allelic loss in an eccrine porocarcinoma. One eccrine porocarcinoma/undifferentiated adnexal carcinoma showed prominent microsatellite instability, probably reflecting an underlying defect in DNA mismatch repair. Overexpression of erbB-2 was observed in three tumors. The low frequencies of LOH and p53 alterations in sweat gland carcinomas contrasted with the multiple genetic defects normally observed in SW, which may be partly explained by the relative protection of cutaneous appendages from ultraviolet light and other environmental mutagens.

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Longitudinal Investigation of Expanded T Cells in Cutaneous T Cell Lymphoma and Related Dermatoses by Genescan Analysis of the T Cell Receptor-γ Rearrangements

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Genescan analysis of PCR amplified T cell receptor (TCR) γ genes identifies T cell expansions according to the distribution of the TCR gene size. Usually a Gaussian distribution of the sizes is observed, but in cutaneous T cell lymphomas (CTCL) a dominating peak demonstrates a clonal T cell proliferation. The present study was aimed to investigate the dynamics of expanded T cell clones in the course of CTCL and related dermatoses.

Five cases of CTCL (mycosis fungoides, MF, n = 2; Sézary's syndrome, SS, n = 2; small cell pleo-morphic CTCL, spCTCL) and two cases of CTCL related dermatoses (unclassified erythroderma, UED; small plaque parapsoriasis, SPP) were included into the study. Per patient, up to 21 blood and skin samples, collected in a period of 4 y, were investigated. Blood and skin specimens of each CTCL patient were characterized by the persistence of a specific peak over the time. While analysis of skin and blood in SS and spCTCL patients revealed a constantly high peak-to-background ratio, a varying ratio was observed in the MF blood samples which corresponded inversely to the extent of the skin lesions. Investigation of all SSP and UED skin specimens revealed a Gaussian distribution, but the blood profiles were characterized by the persistence of several peaks with varying peak-to-background ratio. Nonetheless, these variations could not be correlated to the clinical parameters.

Our findings demonstrate the long-term persistence of the dominating T cell clone in blood and skin of the investigated CTCL patients. With regard to the CTCL entities, SS and spCTCL are characterized by a constantly high amount of clonal T cells in both compartments, whereas in MF a varying ratio of clonal to nonclonal T cells was observed. Since the ratio correlated inversely to the extent of the skin lesions, this might reflect an increased amount of reactive (nonclonal) T cells during extensive skin disease but also a shift of the neoplastic (clonal) T cells to the blood compartment during minimal skin disease. Interestingly, our study demonstrated the long-term persistence of several peripheral blood T cell clones in the UED and SPP patient. Since these clones were not found in the skin samples, their relation to UED and SPP remains unclear but might be a sign of a response against a persisting antigen.

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Analysis of the p14arf-p53 and p16inka-Cdk4 Pathways in Skin Squamous-Cell Carcinomas from Xeroderma Pigmentosum Patients

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We recently demonstrated that deregulation of the p16inka-Cdk4-Rb pathway might occur through *p16inka* UV-induced mutations in human sporadic SCCs. In this study, we explored the p16inka-Cdk4 and the p53-p14arf pathways by searching for *p16Inka*, *p14arf* and *Cdk4* mutations in a set of 17 SCCs from unrelated XP DNA repair patients with a known p53 mutation status. The entire coding sequence of the *INKA-ARF* locus and exon 2 of the *Cdk4* gene (coding for the P16INKA binding domain) were examined by PCR-SSCP. Eight mutations of *INKA-ARF* were detected in five different tumors (29.4%): two in exon 1 α, and six in exon 2, with two tumors harboring multiple mutations. No mutation was observed in exon 1 β of *p14arf*, and in exon 2 of *Cdk4*. The mutations included four tandem CC→[captau][captau] mutations, three C→T mutations (two at a CC site), and one base deletion (C). Six out of eight (75%) mutations were clustered at two codons (58 and 114) of p16INKA. One tumor harbored two mutations in exon 1 α: (i) a C deletion resulting in a 24 aminoacids truncated P16INKA, and (ii) a C→T transition 25 bp upstream of the first ATG site. Predictable effect on P14ARF was only seen in 3 tumors, with an identical missense mutation (Leu→Pro) at a nonconserved interspecies codon. Out of the six tumors harboring a mutated p53, four (67%) also carried a *p16inka* mutation. Our data confirm the presence of UV induced mutations in the coding sequence of the *INKA-ARF* locus in XP SCCs, which appear to affect mostly the *p16Inka* gene, rather than the *p14arf* gene. Furthermore, *p16inka* mutations seem to occur at specific hot spot codons, and some tumors may harbor multiple mutations. Finally, in contrast to sporadic SCCs, *p16inka* and *p53* inactivation in XP tumors could have cooperative effects in the tumorigenic process.

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Chromosomally Verified Malignant Cells are Double Positive for CD4/CD8 and CD45RA/CD45RO in Sézary Syndrome

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T-cell clones in mycosis fungoides (MF) and Sézary syndrome (SS), the most common forms of cutaneous T-cell lymphomas (CTCL), usually represent the phenotype of mature CD4-positive cells, with phenotype CD3+,CD4+,CD45RO+, CD30–. We recently identified, with comparative genomic hybridization and ISH clonal chromosomal aberrations in SS both in peripheral blood and skin tumor. We elucidated the phenotype of such cells, with a combinatory, three colour method of immunophenotyping and DNA *in situ* hybridization in two patients. Native blood lymphocytes and touch preparations of malignant lymph nodes and skin infiltrates were immunostained with antibodies to CD3, CD4, CD45RO, CD45RA, CD8 and signaling lymphocytic activation molecule (SLAM). The malignant cells with clonal chromosomal aberrations were brightly CD3+,CD4+ in all tissues studied. They were positive for CD45RO+,CD45RA+,SLAM+,CD8+, so that bright forms predominated in lymph node and skin, and dull in blood. This pattern was not observed in healthy individuals or methodological control preparations. The coexpression of $CD45RO^{bright}/CD45RA^{bright}$ may correspond to the transitional stage from RA+ to RO+, normally observed in secondary lymphoid organs. This is the first demonstration of T-cell differentiation markers in chromosomally verified malignant cells in CTCL.

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Gli1 Protein is Expressed in Basal Cell Carcinomas and Outer Root Sheath Keratinocytes

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Activation of the Sonic hedgehog (Shh) signaling pathway plays a central role in the formation of normal hair follicles and in the development of basal cell carcinomas. The Gli family of transcription factors mediate responses to Shh and we have previously shown that Gli1 mRNA but not Gli3 is differentially expressed in BCCs. In Drosophila the Gli homolog Cubitus interruptus (Ci) interacts with a cytoplasmic multiprotein complex which plays an important role in the regulation of Ci activity and intracellular localisation by proteolytic cleavage. The aims of this study were firstly to determine if Gli1 protein is expressed in BCCs and secondly to look for evidence of post translational regulation of Gli1 activity in Gli1 expressing cells. Using antibodies to epitopes on the N and C terminal regions of Gli1 we demonstrate that Gli1 protein is present in BCCs and that the protein is mainly localised to the cytoplasmic compartment. Cells transfected with epitope tagged Gli1 showed positive staining of both the cytoplasmic and nuclear compartments and we were able to confirm that nuclear localisation is not dependent on C terminal cleavage. Immunostaining of hair bearing skin with the Gli1 antibody showed strong cytoplasmic staining in the outer root sheath keratinocytes. These results show that Gli1 protein is expressed in both BCCs and normal hair follicles. The cytoplasmic localisation of Gli1 in BCCs and ORS keratinocytes and the presence of both nuclear and cytoplasmic staining in transfected cell lines suggest that regulation of the intracellular distribution of Gli1 may also contribute to control of Gli1 activity in human skin.

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Three Different Mutations of *PTEN* Gene in Cowden Disease

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Cowden disease (CD) is an autosomal dominant disease characterized by the association of mucocutaneous lesions, and hamartomas or neoplasia involving thyroid, breast and gastrointestinal tract. CD gene was recently identified as *PTEN*, a putative tumor suppressor gene. We report five families including seven patients with CD in whom *PTEN* mutations were sought. The nine *PTEN* exons were amplified and sequenced using appropriate primers. In family 1, genetic analysis revealed a missense point mutation located in exon 5 at codon 130 (G to T substitution in nucleotide 1193: Arg to Leu alteration). In family 2, a missense mutation was located in exon 1 at codon 13 (A to G substitution in nucleotide 841: Lys to Glu alteration). In family 3, the first nucleotide of intron 2 was changed from G into C causing a mutation in the splice site sequence (IVS2 + 1G→C). Northern blotting confirmed this mutation showing mARN of two different sizes. No mutation was identified in the two last families. The R130L mutation demonstrated in patient 1 was thought to alter the dual-specificity phosphatase site of PTEN protein. The K13E mutation was associated with a mild CD phenotype and no mutation has ever been reported in exon 1. These data suggest that *PTEN* gene mutations observed in CD patients may be involved in carcinogenesis.

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Mucosal Oncogenic Human Papillomaviruses and Extragenital Bowen's Diseases

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Background. Genital Bowen's diseases are known to have a strong association with HPF type 16. On the other hand, previous studies on extra-genital Bowen's disease (EBD) using different hybridization techniques have displayed discordant results for the detection of mucosal oncogenic HPV.

Methods. 94 samples of EBD from 78 patients were investigated clinicopathologically. DNAs extracted from fixed and embedded tissues were analyzed for the presence of the main mucosal oncogenic HPV types 16, 18, 31 and 33 using polymerase chain reaction (PCR) with the specific primers described by Baay *et al* (1) particularly well adapted to fixed tissues and giving small amplimers. Moreover, 11 EBD of the hands were investigated by an situ hybridization ISH) approach.

Results. Out of the 94 extragenital BD obtained from 78 patients, HPV DNA type 16 was detected in 78 cases (83%) from 65 patients (83.3%) by PCR. Nine patients with BD of the hands (90%) had HPV type 16 ans ISH displayed a diffuse hybridization pattern corresponding to the episomal viral form of HPV DNA.

Conclusions. The present retrospective work on 94 samples clearly demonstrates the high prevalence of HPV type 16 infection in EBD and especially in BD of the hands. In our work no specific clinical, topographic or histopathologic features were found in any lesions to be indicative of the presence or the absence of HPV.

References. (1) Baay MFD *et al*: *J Clin Microbiol* 34:745–747, 1996

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Loss of Chromosome 14q is Selected in a Subset of Primary Malignant Melanomas at the Stage of the Vertical Growth Phase

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Primary malignant melanomas are characterized by a high degree of genetic heterogeneity which can be used to detect specific genetic alterations selected during early steps of melanoma progression. Our previous studies suggested that loss of 14q might be selected together with 9p loss. We have therefore analyzed 5 primary malignant melanomas (Clark level III-V) which harboured loss of heterozygosity (LOH) on 9p by microsatellite analysis at markers D9S162, D9S171 or D9S259 for LOH on 14q using markers D14S288, D14S75, D14S53, D14S51, D14S267. The primary melanomas were microdissected at 2–10 different regions and microsatellite PCR was analyzed by temperature-gradient-SSCP. In all analyzed primary tumors we could demonstrate loss on one or more 14q microsatellite markers in at least one microdissected area. Two melanomas showed that LOH on 14q was selected repeatedly within the primary tumor as we could demonstrate different regions within the tumor where LOH on 14q affected opposing alleles which indicates that this genetic change was strongly selected during melanoma tumor progression. Most losses were clustered near marker D14S288 (14q13–21) or near marker D14S267 (14q32) suggesting two independent putative tumor suppressor regions on 14q.

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Expression of Vascular Endothelial Growth Factor-C in Cutaneous and Lymphonodal Melanoma Metastases

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It is well-established that growth of solid tumours is dependent on angiogenesis and vascular endothelial growth factor (VEGF) has been shown to be involved in tumour angiogenesis both *in vitro* and *in vivo*. Melanoma cells express VEGF together with its high affinity receptors and increase the proliferation rate in response to this factor. VEGF may therefore affect melanoma growth by increasing both vascularization and tumour cell proliferation. Among the endothelial growth factors with structural homology to VEGF, VEGF-C displays the highest specificity for lymphatic endothelium. VEGF-C binds to VEGFR-2, as does VEGF, and is also a ligand for VEGFR-3, a tyrosine kinase receptor which is preferentially expressed in the endothelium of lymphatic vessels. Extracellular proteolytical processing leads to mature VEGF-C which acquires VEGFR-2 binding properties and displays enhanced VEGFR-3 activating capability. In the present study, we have characterized the expression of VEGF-C in cultured human melanoma cells derived from cutaneous and lymphonodal metastases. By RT-PCR and Northern blotting analysis VEGF-C mRNA was detected in one of six cutaneous metastases *versus* five of six lymphonodal metastases. On the other hand, normal melanocytes did not express VEGF-C mRNA. Synthesis, secretion and processing of VEGF-C in melanoma cells were also detected by immunoprecipitation analysis. The expression of VEGFR-2 and VEGFR-3 as possible mediators of an autocrine effect of VEGF-C on tumour growth was then investigated. By RT-PCR VEGFR-2 mRNA was detected in 10/12 melanoma cells, while only three out of 12 melanomas expressed VEGFR-3 mRNA. These findings suggest a positive correlation between VEGF-C production by melanoma cells and tumour ability to metastasize via the lymphatics.

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Inducible Nitric Oxide Synthase Expression in Cutaneous T Cell Lymphomas Before and After Treatment with Roferon (Interferon-Alpha-2a)

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Interferon-alpha is a cytokine that is effective in the treatment of a variety of cancers, including nonmelanoma skin cancers like cutaneous T-cell lymphomas (CTCL). Interferon-gamma is a cytokine secreted by activated natural killer and T-cells which stimulates iNOS protein expression in keratinocytes and which is largely produced in CTCL.

So, the aim of this work was to determine if iNOS protein was expressed in CTCL and whether a correlation exists between the level of iNOS protein expression and stage of the CTCL or degree of disease's remission under interferon-alpha therapy.

We examined 14 cutaneous T-cell lymphoma before and after treatment with interferon alpha-2a (Roferon, Roche, France) (five stage Ib, four stage IIb, five stage Sezary) for iNOS protein expression by immunohistochemistry using the peroxidase technique.

The results show that iNOS protein expression was not correlated with the stage of cutaneous lymphoma but rather with the degree of clinical remission. Indeed before treatment, the expression of iNOS in epidermis was variable, varying from a weak to a strong labelling of suprabasal layers without any relation with clinical stage. Under interferon-alpha, no modulation of iNOS expression was detected when patients' state was stable, a down-regulation was observed in case of partial ($\geqslant$50%) or full remission and an up-regulation was observed when cancer get worse.

Our results indicate that the clinical efficiency of interferon-alpha in cutaneous T-cell lymphomas may, in part, be related to the ability of this cytokine to downregulate inducible nitric oxide synthase expression induced by interferon-gamma.

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CDKN2A Methylation as a Predisposing Factor for Familial Melanoma

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Several lines of evidence such as the ability of 5-methylcytosine residues to act as mutation "hot" spots, the high binding affinity of methyl groups for carcinogens, the influence of pre-existing methylation on the *de novo* activity of DNA methyltransferase and the transcriptional silencing of tumor suppressor genes following an aberrant methylation of the associated 5'CpG islands, have prompted us to investigate DNA methylation as a predisposing factor for tumor development. Familial melanoma was chosen as experimental model and the CDKN2 A gene, which harbors the CpG island, was investigated for the methylation pattern. CDKN2 A mutations have indeed been identified in 20%–50% of melanoma kindreds. Affected and unaffected members of seven melanoma families were included in the study. The PCR methylation assay, relying on the inability of some restriction enzymes (*HpaII, CfoI, SacII*) to cut methylated sequences, was used to analyze the methylation status of the first and second exon of the CDKN2A gene. DNA digested before PCR amplification with the methylation-insensitive endonuclease *MspI* and the fragment amplified from undigested genomic DNA were used as controls. Our data show a *de novo* anomalous methylation of the *HpaII* restriction sites in the first exon of the CDKN2A gene in two of seven melanoma kindreds examined. The CDKN2A aberrant methylation profile is present in both affected and unaffected members of the same melanoma family suggesting that an alternate process of DNA methylation of the CDKN2A CpG island may represent a predisposing element for this tumor.

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Distribution of Connexins 26 and 43 in Basal Cell Carcinoma, Epidermal Squamous Cell Carcinoma and Precursor Lesions

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Changes of intercellular communication via gap junctions may be required for tumour progression. Connexin 26 (Cx26) is a gap junction protein which can suppress tumour formation. Our previous work showed that Cx26 was absent from normal interfollicular epidermis but present in differentiating keratinocytes in benign hyperplasia. We now describe the distribution of Cx26 and Cx43 in basal cell carcinoma (bcc), epidermal squamous cell carcinoma (scc), Bowen's disease, actinic keratosis and keratoacanthoma. Cryosections of tumours and adjacent skin were stained with antibodies for Cx26 and Cx43 and examined by confocal fluorescence microscopy. Proliferating cells were identified with antibodies to Ki67, apoptotic cells by the tunel method. Cx26 and Cx43 were seen as discrete plaques at points of cell contact, mainly in nonproliferating, tunel-negative cells, within epidermis overlying bcc and within bcc nodules. Cx26 was also found on the basal laminar surface of peripheral cells in bcc, while Cx43 was increased in stromal cells surrounding bcc. Cx26 appeared in acanthotic epidermis of actinic keratoses, Bowen's disease, in keratoacanthomas and in well differentiated regions within scc. However, Cx26 was absent from cells at the invading edges of squamous cell carcinomas. In areas of dysplasia Cx26 was often redistributed from membrane into a perinuclear location. In Bowen's disease and scc there was a patchy loss of Cx43 staining, particularly at the invading edges of scc. The distribution of Cx26 and Cx43 within bcc nodules is consistent with a follicular outer root sheath phenotype, although the significance of Cx26 plaques at the basal lamina is unclear. Up regulation of Cx26 in benign hyperplasia followed by progressive loss of Cx26 and Cx43 in Bowen's and scc supports a role for loss of gap junctional intercellular communication in progression of scc.

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A High Incidence of Sebaceous Carcinoma Associated with Microsatellite Instability in Renal Transplant Recipients

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Sebaceous carcinomas (SCs) are rare appendageal tumours predominantly seen in association with Muir–Torre syndrome (MTS), an inherited disorder of DNA mismatch repair. Ten SCs were identified from 160 000 patient entries in the histopathology database at the Royal Hospitals Trust (RHT) between 1989 and 1999. These comprised five tumours in four patients from a cohort of 600 renal transplant recipients (RTRs), one tumour from a patient known to have MTS, and four presumed sporadic cases. We analysed nine SCs for microsatellite instability (MSI) including five tumours from five RTRs (four RHT patients and one other), one MTS associated SC and three sporadic cases. Tumour DNA extracted from microdissected paraffin-embedded sections was PCR amplified at five or more microsatellite regions. Major MSI was observed in three of five transplant-associated tumours and in the one case of MTS but was not found in any of the three sporadic SCs. The high incidence of SC RTRs, together with the demonstration of MSI in a proportion of these tumours, suggests that immunosuppression may unmask a mutator phenotype in a subset of patients. Four out of five RTRs with sebaceous carcinomas also had multiple squamous cell carcinomas and two also had gastrointestinal neoplasms. To what extent this mechanism contributes to other malignancies in transplant recipients remains to be established.

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Expression of HLA Class 1 Molecules in Primary Malignant Melanoma Lesions in Relation to Tumour Thickness and Metastasising Activity

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Tumour thickness is the most important prognostic factor in malignant melanoma (MM). In general thin lesions do not metastasise and have a good prognosis, while thick lesions often do, which reverses the prognosis. This may be due to a change in the immunological profile of the tumour. We studied the expression of HLA-class 1 molecules in 32 primary MM lesions by immunohistochemistry. The tumours were classified on the basis of two criteria – thickness and development of metastases. Fifteen lesions were thinner than 0.76 mm and 17 were thicker than 1.5 mm. In 19 of the patients (10 with thin and nine with thick primary tumours) no metastases developed in the follow up period (10.5 $\pm$ 1.8 y). In 13 patients (five with thin and eight with thick primary tumours) metastases did develop and the median survival of this group was 3.5 $\pm$ 2.8 y. Five of the examined MM specimens were completely negative, 12 demonstrated a 50% or higher loss, eight yielded a heterogeneous staining pattern and only seven showed a strong homogeneous staining. Five specimens with normal HLA class 1 expression belonged to the group of melanomas with thickness smaller than 0.76 mm and no metastases and another was thicker than 1.5 mm but never metastasised. Further a correlation was detected between the expression of HLA-class 1 and the presence of an inflammatory cell infiltrate. Thus loss of HLA-class 1 expression in primary melanoma lesions is related to a diminished immune response of the organism and seems to indicate a bigger metastasising potential.

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Malignant Melanoma Diagnosis by Raman Spectroscopy and Artificial Neural Network

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NIR-FT Raman spectroscopy (NIR-FT RS) is a nondestructive method enabling analysis of the molecular structure.[1,2] Due to neoplastic transformation detectable changes in chemical structure of proteins, lipids and other compounds often take place. NIR-FT Raman spectra of malignant melanoma (MM), basal cell carcinoma (BCC), seborrhoeic keratosis (SK), pigmented nevi (NV) and normal skin (NOR) differ from one another.[3,4] Thus NIR-FT RS may aid in skin cancer diagnosis. NIR-FT RS spectra are complex and difficult to analyse manually. Here, we used artificial neural network (ANN) for rapid classification of Raman spectra of malignant melanoma. 20 MM, 45 BCC, 21 SK, 41 NV and 50 NOR Raman spectra were collected from skin biopsies and used for neural network training (80%) and evaluation (20%). The used ANN is a two layer feed-forward network followed by a modified Softmax normalisation to interpret the output as probabilities. To reduce the number of ANN inputs without information loss, principal components analysis (PCA) was used on the Raman spectra.

The sensitivity and specificity of malignant melanoma diagnosis were 89% and 84%, respectively. In conclusion, NIR-FT Raman spectroscopy with artificial neural network analysis is a promising tool for the rapid, automatic diagnosis of skin cancer.

1. Gniadecka *et al.*: *J Invest Dermatol* 110:393–398, 1998; 2. Gniadecka *et al.*: *J Invest Dermatol* 111:1129–1133, 1998; 3.Gniadecka *et al.*: *J Raman Spectroscopy* 28:125–129, 1997; 4. Gniadecka *et al.*: *Photochem Photobiol* 66:418–423, 1997.

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Inducible Nitric Oxide Synthase Expression in Basal and Squamous Cell Carcinomas

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Nitric oxide synthesized by the enzyme iNOS and found in neoplastic tissues has been implicated in the development of tumor by different mechanisms, including angiogenesis, immunosuppression, mutagenicity, cytotoxicity. No study was performed concerning the relationship between the level of iNOS expression in tumor cells and the state of differentiation of carcinomas.

So, the aim of this work was to investigate the iNOS protein expression in basal and squamous cell carcinomas. For these last ones we determined if there was a correlation between the level of iNOS protein expression and the state of differenciation or the state of immunosuppression (kidney transplants).

We studied 15 basal cell carcinomas and 27 squamous cell carcinomas (six carcinomas *in situ*, seven carcinomas slightly and moderatly differentiated, seven carcinomas very differentiated, seven carcinomas in patients with a kidney transplant) for iNOS protein expression by using immunohistochemistry with a peroxidase technique.

The results shew that iNOS protein expression in patients with basal cell carcinomas was moderate to strong in suprabasal layers of epidermis absent or very weak in tumor cells whereas it was weak or moderate in suprabasal layers of normal human epidermis. In squamous cell carcinomas, there was no difference between patients with or without kidney transplant. Indeed, the expression varied from a weak to a strong labelling in suprabasal layers of epidermis For 25% of carcinomas slightly to very differentiated, the expression was more stronger in epidermis far from the tumor than in epidermis above the tumor. The iNOS expression was null or very weak in tumors except for 7% of cases where it was moderate.

In conclusion, the low iNOS expression in tumor cells may be an escaping way of tumoral cells to apoptosis normally induced by nitric oxide.

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Immunohistochemical Study of Caspase 3 Expression in Basal and Squamous Cell Carcinomas of the Skin

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Apoptosis is an important biologic process occurring both in normal tissues and pathologic conditions, including neoplasia. Caspase 3 (CPP32, apopain) is a member of the interleukin-1-β converting enzyme (ICE) or cell death effector (ced-3) family, which is involved in the induction of apoptosis. CPP32 seems to be the most directly correlated with apoptosis because of its downstream location in the proteases cascade pathway. No data exist so far on the involvement of CPP32 in human skin tumors. In order to gain insight into the role of CPP32 in cutaneous carcinogenesis, we investigated immunohistochemically the expression of CPP32 in formalin-fixed, paraffin-embedded sections of normal skin, basal (BCC, *n*:22) and squamous cell carcinomas (SCC, *n*:17), using a specific polyclonal antibody. In normal skin, CPP32 was expressed in the cytoplasm of keratinocytes of the epidermis, hair follicles, sebaceous and sweat glands. Western blotting performed on protein extracts of epidermal keratinocytes showed a band of 32 kDa MW, confirming the specific expression of CPP32 in the epidermis. In skin tumors, the expression of CPP32 was semiquantitatively scored using with reference to the labeling intensity of the overlying epidermis (1: equal, 0: lower, 2, 3: higher). CPP32 was found expressed in the cytoplasm of tumor cells in SCC (mean score 1.176 ± 0.86) and much more weakly in BCC (mean score .363 ± 0.57). The peritumoral infiltrate and endothelial cells of the dermis surrounding tumor masses (but not in human skin) often expressed CPP32. These results, showing a regular expression of CPP32 in epithelial cells of normal skin and a differential one in BCC and SCC, suggest that CPP32 has a potentially important role in the biology of both normal human epidermis and the tumors arising therefrom. In the latter, the pattern of expression of CPP32 is inversely correlated to that of *bcl2* (an oncogene protecting cells from apoptosis), known to be expressed in BCC but not in SCC. Further studies are in progress in order to unravel the precise role of CPP32 in cutaneous carcinogenesis.

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Serum p53 Antibodies in Patients with Skin Carcinomas

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Circulating p53 antibodies have been detected in patients with various p53-mutated cancers. These antibodies are tumor but not organ specific and can be of prognosis or predictive values in certain tumors and can therefore serve as seric tumoral markers. Because skin carcinomas have a high incidence of p53 gene mutations which are detectable at early tumoral stage (in precancerous lesions) we have investigated the presence of serum p53 antibodies by using a commercially available enzyme-linked immunosorbent assay-kit (anti-p53 ELISA, Pharmacell, France) in a series of 88 histologically proven-skin carcinoma patients in comparison with a sex and age matched control group (skin carcinoma-free inflammatory dermatosis, N = 129) after informed consent. Additionally, P53 protein stabilisation was studied by immunohistochemistry in a proportion of the tumors (N = 38) to confirm for p53 gene alteration in our series of patients. Our results showed a non significant and low incidence (3, 5%) of p53 antibodies in our skin carcinoma group of patients in comparison to the control group (1,5%) although P53 protein overexpression could be confirmed *in situ* in more than 76% of tumor studied. These paradoxical results according to observations made in other cancers of epidermoid origin (i.e., oesophagal or lung cancers) demonstrate an important variation in the value of such antibodies among this group of tumors. Because most skin carcinomas are developed on sun-exposed body sites, we hypothesize that UV-induced immunosuppression could be partly responsible for this low anti-P53 serum immunoreactivity in these patients. Additionally, the immunogenicity generated by mutated p53 could vary with the type of mutation observed. These results clearly show that p53 antibodies can not serve as reliable markers in high risk skin cancer groups of patients, and suggest that p53 gene inactivation and protein stabilisation are not sufficient to induce a circulating anti-p53 reactivity.

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Clonal P53 Mutated Patches in Normal Sun Exposed Skin do not Predispose to Multiple Skin Carcinomas

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Two distinct patterns of immunostaining for P53 have been demonstrated in normal sun-exposed skin: a diffuse pattern of wild type (WT) P53, and an intense compact pattern corresponding to a mutated P53 protein. The compact staining has been referred as P53 patches, present in 65% of the patients on sun-exposed skin. The relationship between these P53 patches and skin cancer formation (basal or squamous cell carcinomas, BCC and SCC, respectively) is not clear and some authors have interpreted them as having very small or even no precancerous potential whereas others have proposed them as cancer-prone, non cancerous mutant cells awaiting further genetic hits. To further inquire the relationship between P53 patches and skin cancer, we compared patients (mean age 71 y) with either multiple (N = 129; 75 BCC, 34 SCC) or single (N = 122; 91 BCC, 31 SCC) tumors on sun exposed area for presence of patches in non tumoral skin. Patches were detected by immunohistochemistry on paraffin-embedded tissue sections using the DO7 anti-p53 antibody. All slides were red blindly by 3 independent observers. P53 patches were detected in 67% of sun-exposed normal skin confirming the incidence reported in the literature in patients over 50 y of age. However there was no significant difference in P53 patches incidence neither between multiple or single tumors nor between SCC and BCC whatever the age (before 40 y, before 50 y or after 60 y). Moreover P53 patches were not associated with sign of dysplasia, whereas the presence of solar elastosis was found in most cases. These results strongly support that p53 patches are UV-related epiphenomenons independent of skin cancer development.

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Germline and Somatic Mutations of the CDKN2a Gene in a Patient with Multiple Melanomas

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The development of multiple melanomas may be correlated either to environmental factors such as ultraviolet light or to a genetic susceptibility. In support of this latter hypothesis, it is noteworthy that 8% to 24% of patients with more than one melanoma may have family histories of melanoma. In addition, the primary lesion in patients with multiple melanomas develops at a relatively early age. Germline mutations of the CDKN2a, which encodes the $p16^{INK4a}$ cell cycle regulator, have been identified in a high percentage (up to 50%) of patients with familial melanoma. In order to clarify the presence of genetic predisposition to the development of multiple melanomas, we analyzed germline and somatic mutations of the CDKN2a gene in a 35-y-old patient with seven melanomas and no family history. We used polymerase chain reaction and direct DNA sequencing to identify germline mutations in the CDKN2a gene. Moreover, microsatellite instability (MSI) and loss of heterozygosity (LOH) at 9p21 (D9S974, D9S126) were examined in each tumor in laser microdissected tumor sections by amplification of (CA)*n* repeat units, electrophoresis of the PCR product and hybridization with ^{33}P-end-labeled oligonucleotides. Analysis of genomic DNA showed a G→T transversion in exon 2 of the CDKN2a gene, which results in a glycin (GGG) to a tryptophan (TGG) substitution at codon 101 of $p16^{INK4a}$ protein. Such mutation has been identified in the patient as well as in one of the two unaffected daughters. In addition, LOH at D9S974 has been detected in two of seven melanomas and MSI at D9S126 has been found in one of seven melanomas. Based on our results, the presence of a germline mutation of the CDKN2a gene is highly suggestive of a genetic predisposition to the development of multiple melanomas. Finally, detection of CDKN2a somatic alterations in the tumors further support the pathogenetic role of bi-allelic inactivation of CDKN2a in melanoma.

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Homozygous Deletions of CDKN2 and PTCH Genes in Nonmelanoma Nonfamilial Skin Tumours

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Loss of heterozygosity of chromosome 9p and 9q alleles is a frequent event in skin tumours. Recently, two tumour suppressor genes on chromosome 9 were identified, CDKN2A and PTCH genes. The CDKN2/p16/MTS1 gene is located in 9p21 and encodes a p16 nuclear protein that acts as an inhibitor of cyclin dependent kinase 4 and regulates the G1/S transmission of the cell cycle. The homozygous deletions of CDKN2 gene occur in many types of human tumours and this gene has been shown to be a familial melanoma gene.

The PTCH gene (locus 9q22.3) is a human homologue of Drosophila segment polarity gene named patched and has been shown to be a familial nevoid basal cell carcinoma syndrome gene. In our study we analyse the frequency of homo-and hemizygous deletions of CDKN2A and PTCH genes sequences in 27 nonmelanoma skin tumours derived from 26 patients.

The genomic DNA from peripheral blood as well as from fresh tumour tissue was isolated in each case and the sequences of two exons of CDKN2 (ex. 1 and 2) and three exons PTCH (exon 3, 6, and 15) genes were amplified. Among 27 analysed tumours one showed the histological features of keratoacanthoma (KA), 21 basal cell carcinoma (BCC) and five squamous cell carcinoma (SCC). PTCH gene deletions were found in one of one KA, two of five SCC and eight of 21 (38%) BCC cases. CDKN2 gene deletions were found in one of one KA, two of five SCC and six of 21 BCC cases. In BCC the deletions of PTCH gene were more frequent in more clinically advanced tumours (T3-T4) than in the ones with lower stage. Our results confirm the high frequency of PTCH deletions in BCC and indicate that deletions of CDKN2 gene are also frequent events in either BCC or SCC of the skin.

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Complex Cytogenetic Aberrations in Two Patients with Sezary Syndrome

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Sezary syndrome (SS) is a rare non-Hodgkin lymphoma with a T-cell phenotype, with cutaneous involvement as its predominant feature. The cytogenetic literature on chromosomal aberrations in SS is limited and contains the description of clonal chromosomal aberrations in about 72 cases. No specific aberrations have been shown yet. In mostly complex, chromosomal aberrations the chromosomes 1, 2, 6, 17, 14 and 11 are most frequently involved.

In our study we report the results of cytogenetic examinations performed on two patients with Sezary's syndrome. The chromosomes were obtained from peripheral blood cells cultured for 72 h with phytohaemagglutinin stimulation. The metaphases were examined with a GTG staining technique. Patient D.R. has had one examination, and patient B.K. has had two cytogenetic analyses in 16 mo.

Both patients have had abnormal karyotypes with multiple structural and numerical chromosomal changes. Two consecutive examinations of patient B.K., performed within 16 mo, revealed evolution of the karyotype with the complication of chromosomal changes. The complexity of the chromosomal aberrations correlates with clinical progression of the disease.

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Transformation to High Grade Lymphoma is not Prevented by Extracorporeal Photopheresis

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Extracorporeal photopheresis (ECP) is the preferred treatment of leukemic small cell CTCL (Sezary). It recently has been suggested that ECP acts throw the stimulation of a specific immune response against expanded malignant clonal T-cells. On the other hand, it can not be regarded as a curative therapy for low grade CTCL and it even has recently been suggested, that it does not prolongs survival in patients with Sezary syndrome. Further more in three patients cytological transformation to a large cell lymphoma (more than 50% large cells) was observed during ECP therapy. Nothing is known about the development of the CD30 positive large cell phenotype during ECP therapy, witch is a special differentiation of either primary CTCL or secondary due to transformation. We here report on a case of CD7 positive CD4 positive Sezary CTCL who after 11 curses of ECP developed a rapidly growing nodal involvement of CD30 positive large cell anaplastic lymphoma. Our observation stresses the significant of recent reports showing that large cell transformation although to large cell CD30 positive anaplastic lymphoma is not avoided by ECP-therapy. The immunomodulating effect of ECP be it modulating of the cytocine pattern, be it induction of apoptosis is not sufficient to prevent further transformation of a low grade malignant T-cell clone.

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The Antiangiogenicactivity of Vitamin D3 is Enhanced by Ketoconazole and its Derivatives

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Ketoconazole is known as a broad spectrum antifungal agent with an anti-inflammatory activity. Some of its other possible applications (e.g., in policystic ovary syndrome, prostate cancer, as a "cyclosporin-sparing agent" by the immunosuppressive therapy) depend mainly on its effect on the metabolism of various drugs and hormones through the cytochrome P450.

The antineoplastic activity of vitamin D3 has been already shown. It affects cell proliferation, differentiation, and cytokine production, and is a potent regulator of macrophage function.

The aim of the study was to find out whether ketoconazole and whether this activity is enhanced by the combination with vitamin D3 analogues. Tumor angiogenesis (TA) was induced in X-ray immunosuppressed Balb/c mice by i.d. injection of 25×10^4 of HPV16-harboring SKv tumor cells. The systemic i.p. treatment of mice with ketoconazole or calcitriol (active form of vitamin D3) alone led to a significant inhibition of the new blood vessel formation. The combination of both agents enhanced significantly this effect. Since the systemic application of these agents may result in pronounced toxic side-effects, we studied in the same TA experimental model the effectiveness of vitamin D3 analog (tacalcitol) and imidazole derivative (clotrimazolum) applied topically. In this experiment there was the comparable to that by systemic administration, significant inhibition of TA, more pronounced by the combination of both agents. This is the first report on the antiangiogenic activity of ketoconazole and its derivatives. This effect was found to be enhanced by the combination with vitamin D3. This could be explained by the reported ketoconazole-dependent inhibition of vitamin D3 degradation by cytochrome P450 enzyme: 24-OHase (Kang *et al.*: *J Invest Dermatol* 108:513–518, 1997). Topical application of the studied agents has a comparably strong antiangiogenic effect as the systemic administration, with the advantage of allowing to avoid toxic side-effects of such therapy. The potent antiangiogenic activity may be useful in the treatment of angiogenesis-dependent diseases, including tumors.

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Increased Prostaglandin E2 Production by Ultraviolet Radiation may be Involved in Enhanced Inflammation and Immunosuppression in Xeroderma Pigmentosum Group A Model Mice

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Xeroderma pigmentosum group A (XPA) gene-deficient mice show strong UV-induced inflammation and immunosuppression. Prostaglandin (PG) E2 is well known as an inflammatory mediator and also as an immunomodulator. Topical application of indomethacin inhibited UV-induced inflammation and also abrogated UV-induced local immunosuppression in XPA-deficient mice. Therefore, we predicted that the enhanced inflammation and immunosuppression in XPA-deficient mice would be due to increased production of PGE2 after UV exposure. To confirm our hypothesis, we determined the amount of PG and analyzed cyclooxygenase (COX) mRNA expression in UV-irradiated XPA-deficient mouse skin. The amount of PGE2 in XPA-deficient mice significantly increased at 48 and 72 h after irradiation to the level which was 8- to 15-fold higher than that in wild type mice. Expression of mRNA for COX-2, but not COX-1, timedependently increased after irradiation in XPA-deficient mice. These findings indicate that PGE2 may play an important role in inflammation and immunosuppression following UV radiation. In XP, UV-induced DNA damage in the form of pyrimidine dimers, are not repaired. The excess pyrimidine dimers after UV radiation in the XPA-deficient mouse cells may induce COX-2 expression to promote the production of PGE2, which may stimulate the secretion of IL-10 from keratinocytes.

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Modulation of UVB-Mediated Increase in Collagenase/MMP-1 and Stromelysin-1/MMP-3 Protein Levels by p70 Ribosomal S6 Kinase (p70S6k) Activity in Human Dermal Fibroblasts

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Direct DNA damage and reactive oxygen species (ROS) generated upon UVB irradiation are responsible for a variety of pathological conditions in skin cells. Recently, some studies have implicated ROS and/or direct DNA damage as initiating events in the signal transduction pathway(s) leading to increased expression of specific genes. However, only limited information exists regarding the components of the DNA-damage dependent signal cascade lying upstream of specific target genes. Therefore, the aim of this study was to identify components of the signaling pathway which possibly link UVB-mediated DNA damage and induction of matrix-degrading metalloproteinases (MMP). For this purpose, human dermal fibroblasts were incubated with nontoxic concentrations of the immunosuppressant rapamycin, known to specifically inhibit the DNA protein kinase FRAP, and/or the excision repair inhibitor aphidicolin prior to UVB (280–320 nm) irradiation at defined doses. Data obtained by means of cytotoxicity assays, ELISA technique, immunoblotting, Northern blot analysis, and S6 phosphorylation assays showed that UVB-mediated increase in p70S6k activity was significantly reduced by rapamycin treatment which subsequently resulted in a significant reduction of MMP-1 and MMP-3 protein levels by 60% compared to only UVB-irradiated control cells. Both aphidicolin-pretreated and UVB-irradiated cells showed a significant reduction of the MMP-1 and MMP-3 protein and mRNA levels by 80% compared to UVB controls. Interestingly, rapamycin and aphidicolin had no effect on TIMP-1 mRNA and protein levels after UVB irradiation. By contrast, the IL-1β-mediated induction of MMP-1 and MMP-3 was rapamycin-and aphidicolin-independent. In conclusion, these data indicate a requirement for p70S6k in the UVB-induced signaling leading to the induction of distinct MMPs and, furthermore, suggest that this kinase is a component of an IL-1/membrane receptor-independent, but possibly DNA damage-dependent pathway.

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ICG-Mediated Phototherapy for the Treatment of Solid Tumors

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Using porphyrin derivatives with an absorption at 630 nm Photodynamic Therapy is restricted to superficial skin tumors (<3 mm). Indocyanine green (ICG) with an absorption in the near infrared at 805 nm allowing deeper tissue penetration of light is already clinically approved for diagnostic indications.

In vitro the active intracellular uptake of ICG into cells derived from human skin (HaCaT, SCL-1/2) is inhibited by bromosulphophthalein indicating the involvement of the organic anion transporter protein (OATP). Irradiation using a diode laser (805 nm) yielded an effective light and concentration dependent cell killing which was inhibited significantly adding sodium azide or histidine, specific quenchers of singlet oxygen.

In vivo using the amelanotic hamster melanoma implanted into dorsal skinfold chambers pharmacokinetics revealed a maximal fluorescence intensity in tumor directly after injection (2×2 mg per kg b.w.) as determined by intravital microscopy. Best therapeutic results were obtained irradiating subcutaneously implanted amelanotic melanomas by a diode laser (805 nm, 100 J per cm^2, 0.5 W per cm^2) directly after injection of ICG (2×2.5 mg per kg).

This report shows that solid tumors can be effectively treated by ICG-mediated phototherapy mediated by singlet oxygen.

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Ultraviolet A Radiation (UVAR) Generates Ceramides in Human Keratinocytes Through a Novel, Non-Enzymatic Pathway

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We have recently shown that UVAR-induced keratinocyte (KC) gene expression is mediated through the formation of ceramides from cell membrane sphingomyelin as an initial step. In the present study the mechanism by which UVAR induces ceramide generation was assessed. After metabolic labeling of cells with [^{3}H]palmitic acid, lipid extracts from irradiated normal human KC were analyzed for the formation of ceramides by thin layer chromatography (TLC). Ceramides were generated in irradiated KC in a dose-and time-dependent manner. UVAR-induced ceramide formation was mediated through singlet oxygen ($^1O_2$) generation, because $^1O_2$ quenchers inhibited and a $^1O_2$-generating system mimicked UVAR effects. TNFα and IL-1 are known to induce ceramide-mediated gene expression through activation of sphingomyelinases (SMase) that cause hydrolysis of sphingomyelin and thereby generate ceramides. Accordingly, TNFα or IL-1α induced (4-fold) acid and neutral SMase activity in KC, as was assessed in KC protein extracts that were incubated with C^{14}-sphingomyelin. In contrast, SMase activity remained unaltered when KC were treated with UVAR or $NDPO_2$, although both stimuli induced (3-fold) ceramide formation within the same experiment. In order to test whether UVAR/$^1O_2$ generates ceramides independent of SMase activity, sphingomyelin-containing liposomes were irradiated. In this SMase-free system, UVAR as well as $NDPO_2$ treatment caused the formation of ceramides, as was measured by TLC. Moreover, by employing mass spectroscopy we observed that $NDPO_2$ treatment generated a ceramide pattern (increase in C_{16}, C_{18},C_{24}) in sphingomyelin-containing liposomes identical to that observed in irradiated KC. Our studies indicate the existence of a previously unrecognized, nonenzymatic pathway, by which UVAR/$^1O_2$ initiate the signal transduction cascade leading to increased KC gene expression.

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Induction of Apoptosis in Skin-Infiltrating Mast Cells by High-Dose Ultraviolet (UV) A-1 Radiation Phototherapy in Patients with Urticaria Pigmentosa

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In previous years, the mainstay in the treatment of patients with urticaria pigmentosa (UP) has been photochemotherapy with oral administration of 8-methoxypsoralen and subsequent ultraviolet A radiation exposure (PUVA). PUVA therapy is effective, but does not decrease the number of skin-infiltrating mast cells and cessation of PUVA therapy is therefore associated with a relapse of symptoms after only 6–8 mo in almost all patients. More recently, high-dose UVA-1 phototherapy (340–400 nm; 130 J per cm^2 UVA-1 per exposure) was found to be effective for the treatment of UP (*Lancet* 347:64, 1996). In continuation of these studies we now report that UVA-1 phototherapy differs from PUVA by inducing significantly longer remission periods. High-dose UVA-1 phototherapy induced a prompt (after three exposures) and complete (after 10 exposures) improvement of skin as well as systemic symptoms in 20 out of 20 patients with histologically proven UP. Follow-up studies revealed remission periods of 8 mo in 100%, of 16 mo in 60%, and of 20 mo in 40% of these patients. Immunohistochemical analysis of biopsies obtained prior, during and after UVA-1 phototherapy demonstrated that clinical improvement was associated with a significant decrease in the number of skin-infiltrating mast cells in these patients. Accordingly, after only three exposures, mast cell numbers were decreased by up to 50%. Mast cell depletion was preceded by the induction of apoptosis in these cells, as was demonstrated by employing a double-staining technique allowing the simultaneous detection of apoptotic (TUNEL method) as well as tryptase-positive (α-mast-cell-tryptase Mab) cells. Induction of apoptosis in skin-infiltrating mast cells and subsequent mast cell depletion might thus be responsible for the long-lasting clinical improvement that is induced in UP patients by high-dose UVA-1 phototherapy, indicating that high-dose UVA-1 phototherapy is the treatment of choice for UP.

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DNA-Dependent Protein Kinase Catalytic Subunit is Cleaved during UV-induced Apoptosis

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It has been demonstrated that the protease Caspase 3, a downstream molecule of the CD95 pathway, is activated in UV-exposed keratinocytes and the DNA-PKCS is cleaved by ICE-like protease during apoptosis induced by X-rays, staurosporine and etoposide. Therefore, we study whether the DNA-PKCS is cleaved during UV-induced apoptotis in keratinocytes. We used the well-characterized cloned human keratinocyte cell line HaCaT which carries p53 mutations. UVB-induced apoptotic cells were observed by TUNEL assay and agarose gel electropholesis. Western blot analysis was performed using antibodies against DNA-PKCS. The cleavage occured during UVB-induced apoptosis in HaCaT cells. It suggests that the cleavage is associated with loss of DNA-PK activity. Thus, a functional significance of cleavage of DNA-PKCS may be to prevent rejoining fragmented DNA during apoptosis,thereby promoting apoptotic processes. Although apoptosis was not completely blocked by the Caspase 3 inhibitor, the cleavage of the DNA-PKCS was blocked. These results indicate that the cleavage of DNA-PKCS is necessary but not sufficient for UV-induced apoptosis. Further studies are needed to establish other pathways in the formation of UV-induced apoptotic cells.

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A Rat Systemic Herpes Simplex Virus Infection Model as a Tool for Sunscreen Immunoprotection Studies

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Ultraviolet (UV) radiation can induce sunburn, skin cancer and immunosuppression. Sunscreens are used to prevent these harmful effects. There are, however, many conflicting reports regarding the efficacy of sunscreens in preventing UV-induced immunosuppression. Most of these studies are hampered by the lack of a relevant immunological endpoint. Therefore, we have investigated whether sunscreens can protect against the UV-induced impaired resistance to a systemic herpes simplex virus (HSV) infection, using a rat model. For this study, animals were irradiated daily with 1 MED UVB for seven consecutive days on their shaved backs with or without application of various sunscreens (with organic filters or titanium dioxide (TiO_2), all with sun protection factor 10) or vehicle. After the last irradiation animals were infected intranasally with HSV. Five days after infection animals were challenged with inactivated HSV on the ears. The next day delayed type hypersensitivity (DTH) response was determined. Subsequently, animals were sacrificed and the skin was analyzed for cis-UCA, an important mediator of UV-induced immunosuppression. The first parameter that could be scored visually as an effect of UV on the resistance to systemic HSV infection, were clinical signs of illness, such as paralysis. We found that none of the rats in the nonirradiated group showed signs of illness, in contrast to the UV and vehicle group that showed a high percentage of ill animals (88%). The organic sunscreen provided partial protection (29% ill animals), while the TiO_2 sunscreen afforded complete protection (no animals showed signs of illness). These clinical results showed strong correlation with the DTH responses and cis-UCA values. UV irradiation caused a decrease of the DTH responses and an increase of the cis-UCA percentages, while both the vehicle and the organic sunscreen could not prevent these effects. The TiO_2 sunscreen showed complete protection. Our findings show that this systemic infection model is a suitable endpoint to study sunscreen immunoprotection and that micronized pigment containing sunscreens, probably because of their broad absorption spectrum, are promising candidates to prevent UV-induced immunosuppression.

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UVB Induced Depletion in Storage and Functional Epidermal Vitamin A in Hairless Mouse; Differential Prevention by Topical Pretreatment by Retinol, Retinaldehyde and Retinoic Acid

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Previous studies have shown that exposure of hairless mice to UVB induces a depletion in cutaneous vitamin A, i.e., retinol (ROL) and/or retinyl esters (RE). In the present study we adressed three questions: 1. Do UVB affect both retinol and RE or only RE, which would result in a vitamin A *storage* deficiency; 2. Does pretreatment with either topical ROL, retinaldehyde (RAL) or retinoic acid (RA) prevent such a depletion; and 3. Is cutaneous RA, the ligand for nuclear receptors, either when topically applied or formed from topical precursors such as ROL and RAL affected by UVB exposure, which would result in a vitamin A *functional* deficiency. We measured by RP-HPLC epidermal (E) and dermal (D) contents in ROL, RE, and RA following a single UVB exposure of 1 J per cm^2 in hairless mice treated either with RA, ROL, or RAL 0.05% or vehicle. In vehicle treated animals, UVB induced a decrease of RE in E (530 ± 36 pmol per g before; 100 ± 29 pmol per g after UVB) but not in D (486 ± 55 pmol per g before; 480 ± 64 pmol per g after UVB); ROL content was not affected by UVB exposure in E (97 ± 7 pmol per g before; 74 ± 34 pmol per g after UVB), but increased significantly in D (367 ± 107 pmol per g before; 845 ± 102 pmol per g after UVB). Since ROL absorbs at 325 nm, it is likely that it is protected by cellular retinol binding protein. In animal pretreated by ROL, RAL or RA and exposed to UVB the RE contents were : E 631 ± 58; 1223 ± 72; 835 ± 113/D 318 ± 36; 880 ± 108; 531 ± 60 pmol per g and ROL contents: E 40 ± 9; 47 ± 8; 61 ± 18/D 259 ± 35; 738 ± 139; 341 ± 60 pmol per g, respectively, indicating that previous treatment does prevent *storage* deficiency. RA was under the limit of detection (10 pmol per g) in E and D in both vehicle- and ROL-treated mice; in mice treated with either RA or RAL it was 56 ± 11 and 66 ± 21 pmol per g, respectively, in E but not detectable in D; this content (E) did not decrease following UVB irradiation, which indicates that a single high dose of UVB exposure does not induce epidermal *functional* vitamin A deficiency in animals pretreated with either RAL or RA, probably due to the protective effect of cellular retinoic acid binding proteins.

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Non-Coherent Near Infrared Radiation Induces p53 and p21 Tumor Suppressor Proteins in Human Skin Fibroblasts

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Non-coherent near infrared radiation (700–1000 nm), in physiological doses, protects human dermal fibroblasts from the cytotoxic effects of solar UV and PUVA treatment. This protection appears immediately after IR irradiation, increases in the next 24 h, stabilizes between 24 and 48 h then decreases to disappear after 72 h. The mechanisms of this protection are not known. Since solar UV and PUVA are implicated in skin carcinogenesis, it seemed desirable to understand the molecular mechanisms of the IR-induced protection, as well as its implications in UV mutagenesis. Enhanced repair capacity was one of the possibilities envisaged to explain this phenomenon. As the same level of protection was found in XP-A repair-defficient skin fibroblasts, we decided to look for proteins implicated in repair, to which XP-A cells were not defficient.

Normal human primary skin fibroblasts in confluent monolayers were irradiated with 810 kJ of IR (filtered to remove wavelengths shorter than 700 nm). This dose was delivered in 30 min. At different times after irradiations the cells were examined for induction of p53 and p21 proteins by indirect immunofluorescence and by western blotting analysis, using specif monoclonal antibodies to these proteins. With both methods, the results indicated increase of these two proteins. Increase of p53 was detectable already at time zero after IR, reached a maximum after 6 h and stayed stable until at least 24 h. Increase of p21 was detectable only after 6 h, and was less marked than p53. As these proteins have been shown to be implicated in repair of UV-B induced damage to cells either directly or indiretly (by inducing transcription of other repair-related proteins), we propose that increase of p53 and p21 proteins is at least one of the steps triggering IR-induced protection against solar UV cytotoxicity.

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Increased UVC Induced DNA Damage in Fibroblasts in Pommeranos in Brazil Compared to German Inhabitants

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Pommeranos (P) in Brazil exhibit multiple nonmelanoma skin cancers in young age compared to inhabitants of our town (T). Both groups were studied for DNA-damage after UVC radiation to evaluate if there might be differences in the DNA repair.

After informed consent, 6 mm punch biopsies of normal skin were obtained from P (skin type I,II,III, n = 3) and T (skin type I,II,III, n = 3). Fibroblasts were cultured, irradiated with UVC (1,8 mJ per cm^2, UV Stratalinker 2.400), stopped at 5; 10; 20; 30; 1 h, 2 h, 3 h, 4 h and studied for the repair capacity with gel electrophoresis in the Comet assay (10 gels per person). Comet shaped cells in the immunofluorescence were photodocumented (10 cells from every gel = 100 cells per person), scanned and evaluated with the software (Zero D-Scan, Scanatycs) for the tail moment (product of percentage of DNA in the tail and the distance between the centers of mass of the head and the tail regions). Controls were irradiated, but stopped before repair starts.

In P, the tail moment is higher (I = 53,49; II = 47,21) compared to T (I = 32,91; II = 39,77). In P, repair starts earlier and reaches the maximum earlier (I = 30 min; II = 1 h) compared to the maximum in T (I = 1 h; II = 2 h). In P it takes more time until the repair is finished (I = 4 h; II (4 h) compared to T (I = 3 h; II = 4 h). In P and T skin types III show only minor differences for these parameters. Control fibroblasts showed no tail.

In skin type I,II, fibroblasts of P are more susceptible to UVC damage than fibroblasts of T. In skin type III there is no difference between both groups. Environmental high UV irradiation in P is not the only reason for the increased occurrence of non melanoma skin cancer as the efficacy of nucleotide excision repair plays an additional role.

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Protective Effects of Sunscreening Agents on Photocarcinogenesis, Photoaging, and DNA Damage in XPA gene Knock Out Mice

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We investigated the protective effects of commercial sunscreening agent against UVB-induced skin tumor and photoaging in xeroderma pigmentosum A (XPA) gene-deficient (–/–) mice. XPA wild type (+/+) and (–/–) mice were irradiated at the cumulative doses of 1–2.6 J per cm^2 UVB during the period of 17–24 wk with or without SPF 60 or SPF 10 sunscreening agent. SPF 60-agent afforded stronger protection against photocarcinogenesis in (–/–) mice than SPF 10 sunscreening agent, although not completely. The number and size of tumors developed were significantly smaller in mice treated with SPF 60 material. In order to estimate photoaging, mast cells and elastin were histochemically stained. SPF 60 sunscreen strongly protected against both mast cell infiltration and elastin accumulation than SPF 10 suncreen. In (–/–) mice, strong local and systemic immunosuppression was induced at 20 mJ per cm^2 of acute UVB irradiation compared with (+/+) mice. SPF 10 and SPF 60 sunscreens provided protection against local and systemic immunosuppression, but not completely. We further evaluated the sunscreen protection against UV-induced DNA damage. Cyclobutane pyrimidine dimer (CPD) was observed by immunofluorescent staining with monoclonal antibody. SP17 60 sunscreen showed stronger protection for CPD than SPF 10 sunscreen in (–/–) mice. Our results indicate that sunscreening agent can afford protection against UV-induced skin tumor and photoaging due to inhibition of UV immunosuppression and DNA damage. However, higher SPF agents should be used, because SPF 10-material was not sufficiently protective.

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The Antipsoriatic Effect of Xenon Chloride Ultraviolet B Laser could be Mediated by the Induction of T Cell Apoptosis

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We could show earlier that the 308 nm xenon chloride (XeCl) ultraviolet B (UVB) laser is more effective for psoriasis than the 311 nm narrow band UVB light. Our aim in this study was to investigate whether the XeCl laser induces T cell apoptosis *in vitro* and whether the apoptosis-inducing effect of the XeCl laser depends on the frequency of impulses. Separated peripheral blood mononuclear cells were laser-irradiated, then the apoptotic cells were detected by TUNEL assay or Apo2.7 monoclonal antibody staining. The fluorescence intensity was analysed by flow cytometry. Irradiation with 20–200 mJ per cm^2 dose dependently induced T cell apoptosis detected with both methods. The first apoptotic T cells appeared 12 h after laser treatment measured by Apo2.7 staining. 24 h after irradiating with 66 or 200 mJ per cm^2 the percentage of Apo2.7 positive T cells was 45% or 80%, respectively. The results obtained with TUNEL assay were similar, however, only 55% of the T cells showed TUNEL positivity 24 h after irradiation with 200 mJ per cm^2. No significant change was observed in the extend of apoptosis after irradiating the cells with different impulse frequencies. We have proved that the 308 nm XeCl UVB laser induces T cell apoptosis *in vitro*. These results support the hypothesis that the antipsoriatic effect of XeCl UVB laser could be mediated by the induction of T cell apoptosis.

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UVA Induced NFκB p50/p65 Expression in Hacat Cells can be Decreased by L-Ascorbic Acid

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UVA irradiation of keratinocytes leads to free radical formation which cause lipid peroxidation and cytokine modulation. These effects are known to be inhibited by L-ascorbic acid. Aim of this study was to further elucidate the intracellular signal transduction pathway via NFκB for free radicals in keratinocytes.

Subconfluent Hacat cells partly cultivated under the influence of L-ascorbic acid (10^{-4} mol per liter) for 6 d were UVA irradiated (20 J per cm^2). Free radical formation was measured by production of thiobarbituric acid reactive substances (TBARS). Nuclear extracts were prepared 30 min after UVA irradiation. NFκB expression was determined by electrophoretic mobility shift assay (EMSA), and p50/p65/c-rel subunits were analyzed by supershift assay.

UVA upregulates NFκB expression up to 42%. Activated NFκB consisting of p50 and p65 subunits were expressed, whereas c-rel was negative. NFκB expression was downregulated by L-ascorbic acid up to 66%. TBARS formation was increased after UVA irradiation up to 150% cellular protein. This effect was inhibited by L-ascorbic acid up to 12.5%.

These results demonstrate that signal transduction of UVA induced free radicals in Hacat cells is mediated through NFκB, and this effect can be partly inhibited by L-ascorbic acid.

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Extracorporeal Photoimmunotherapy in Patients with Steroid-Dependent Crohn's Disease

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Extracorporeal photoimmunotherapy (ECP) has been shown to be effective in the treatment of a number of T-cell mediated disease with a steroid-refractory course. In a prospective, open monocentric three phase study of 24 wk we evaluated the safety and efficacy of ECP in patients with steroid-dependent Crohn's disease.

Out of 786 patients screened, 24 with a six month history of steroid-dependent Crohn's disease entered phase I in which standardized prednisolone tapering was attempted in two week intervals. Patients with steroid maintenance–dose (= lowest daily prednisolone dose sustaining a Crohn's Disease Activity Index of less than 200) of at least 10 mg prednisolone at the end of phase 1 were eligible for phase 2 in which ECP was performed on two consecutive days at 14 d intervals. Duration of clinical response after ECP was observed during phase 3. Clinical remission was defined by maintenance of Crohn's Disease Activity Index score of less than 150 after discontinuation of prednislone and clinical response by a 50% reduction of prednisolone maintenance-dose at the end of phase 2. As secondary outcomes changes between phase 1 and 2 in the scores of the Crohn's disease activity index, the quality of life index, serum concentration of C-reactive protein, plasma adrenocorticotropic hormone, and intestinal permeability were considered.

Twenty-four patients entered the trial, eight patients were prematurely withdrawn due to violation of the steroid tapering regimen or for personal reasons. Six patients were successfully tapered below a maintenance-dose of 10 mg prednisolone. Ten patients were included in phase 2. Under ECP four patients (40%) achieved clinical remission after a median time of 20 wk, and four other (40%) clinical response after a median period of 7 wk. Statistical significant reduction in serum C-reactive protein levels ($p = 0.017$), as well as in intestinal permeability ($p = 0.036$) were observed, and an increase of quality of life ($p = 0.008$) and plasma adrenocorticotropic hormone levels ($p = 0.005$). One patient underwent cholecystectomy at week 12 and was withdrawn. Clinical remission remained stable in three of four patients during follow-up, median duration of clinical response was 16.5 wk.

ECP represents a safe and efficacious adjunctive steroid-sparing therapy in patients with chronic active Crohn's disease.

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UV-Induced Production of Immunosuppressive Mediators in Human Skin: Prevention by a Broadspectrum Sunscreen

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Irradiation of the skin with UV-light is well known to cause local as well as systemic immunosuppression. Therefore, the protective effect of a new broad spectrum sunscreen on the UV-induced production of immunosuppressive mediators interleukin-10 (IL-10) and α-melanocyte stimulating hormone (αMSH) was investigated.

The broad spectrum sunscreen used has both a very high sun (SPF > 60) and UVA (UVAPF = 28, Persistent Pigment Darkening method (PPD)) protection factors.

Thirty minutes after the application (2 mg per cm^2) of either the broad spectrum sunscreen or its vehicle control, the volar side of the forearms of four human volunteers were irradiated with UV-light (2 MED) using a solar simulator. Twenty-four hours after irradiation, suction blisters were performed. Using a specific ELISAs blister fluids were analysed for IL-10 and αMSH. Total mRNA was isolated from the blister roofs, reversed transcripted and the resulting cDNA was used for RT-PCR using primers specific for IL-10, αMSH and β-actin.

Whereas, in the vehicle controlled area, the formation of erythema clearly was visible, it was suppressed on the broad spectrum treated area. Moreover, in comparison to untreated skin, IL-10 and αMSH expression were significantly upregulated in UV-irradiated skin both at the protein and mRNA level. Upon treatment with the broadspectrum sunscreen, the αMSH and IL-10 levels in the suction blister fluids were decreased in comparison to the untreated control area. Similarly, mRNA expression of IL-10 and αMSH was downregulated when compared to untreated irradiated skin.

These data provide first evidence for induction of immunosuppressive mediators *in vivo* in the skin upon irradiation with UV-light. In addition, there is evidence that the use of a highly effective sunscreen covering the entire UV spectrum (UVB + UVA) can inhibits the UV-mediated induction of these suppressor factors and thereby may prevent local UV-induced immunosuppression.

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Ultraviolet Radiation Exposure and the Spectrum of Mitochondrial DNA Deletions in Human Skin

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As mitochondrial DNA (mtDNA) is a significant target of ultraviolet radiation (UVR) in human skin we have examined the pattern of mtDNA deletions which may allow its use as a marker of cumulative UVR exposure. Previous studies have focussed on the frequency of single mtDNA deletions alone. We have therefore determined the mtDNA deletion spectrum of almost the entire 16 569 bp-mt genome in relation to sun exposure by using a long PCR technique. We investigated the deletion spectrum in split skin samples from sun-exposed (n = 13 dermis (D), n = 28 epidermis (E)), intermittent (n = 7 (D), n = 8 (E)) and sun-protected body sites (n = 6 (D), n = 9 (E)). There was a significant increase in the number of deletions with increasing UVR exposure in the epidermis (Kruskal–Wallis test, $p = 0.0015$) but not the dermis ($p = 0.6376$). This observation was not confounded by the age associated increase in the number of deletions within (Kruskal–Wallis test, $p = 0.0491$) or between different UV-exposed sites (Spearman's rank correlation, $p = 0.058$). The absolute median number of deletions in the dermal samples was generally greater than the corresponding epidermis suggesting that dermis harbours a higher amount of mtDNA damage. Long PCR appears to represent a qualitative but a more sensitive technique compared to the detection of single deletions alone (deletion frequencies of 96% *versus* 27%, respectively) for determination of UV induced DNA damage in skin.

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Photooxidative Cell Killing *In Vitro* After Photosensitization with a New Porphycene

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9-Acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene (ATMPn) is a promising new photosensitizer characterized by absoprtion around 640 nm and high singlet oxygen yield. To study the mechanism of action *in vitro* intracellular uptake, intracellular localization, photooxidation, cell survival and ultrastructural changes following photosensitization in human cell lines derived from the skin (SCL1/SCL2, HaCaT, fibroblasts) were investigated. Using flow cytometry cellular fluorescence was determined as a marker of ATMPn uptake after incubation for 60 min. Co-staining with fluorescent dyes specific for cell organelles revealed an intracellular localization of ATMPn in lysosomes. Following irradiation using an incoherent light source (580–740 nm) and a light fluence of 24 J per cm^2, phototoxicity was determined by means of the MTT-assay. For all cell lines ATMPn concentrations above 15 nM yielded a significant phototoxic effect. The EC_{50} for SCL1 cells was 11.2 ± 2.9 nM ATMPn. ATMPn uptake and phototoxicity was more effective for HaCaT and SCL1 as compared to SCL2 and N1 cells. Phototoxicity and lipid peroxidation was significantly inhibited using sodium azide, a specific quencher for singlet oxygen. Growth curves confirmed the results of the MTT assay. Immunofluorescence and electron microscopy revealed damage to tonofilaments, plasma membrane, and mitochondria, indicating an apoptosis-unrelated mechanism. A dose yielding complete cell killing as needed for oncologic indications, might lead to necrosis whereas lower sublethal doses result in induction of apoptosis.

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Photodynamic Therapy against Melanoma Cells: High Efficacy of Axially Bound Silicon Phthalocyanines

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Metallated phthalocyanines are currently studied as potential photosensitizers in photodynamic therapy (PDT) of cancer cells. Indeed, a long triplet lifetime can get high yield of $^1O_2$, $O_2^{\circ-}$ and/or °OH involved in cytotoxic effects. Nevertheless, stack aggregation, usually encountered for the planar molecular structures, is responsible for a decrease of triplet lifetime of electronically excited states and, as a result, a low PDT efficacy. We report here the gain brought by the axial linking of hydrophobic cores onto the metal of new synthesized silicon phthalocyanines (SiPc).

bis(tri-*n*-hexylsiloxy)SiPc (HexSiPc), bis(octyloxy)SiPc (OctoSiPc), bis(cholesteryloxy)SiPc (CholoSiPc) and bis (cholesteryloxysiloxy) SiPc (CholSiPc) were synthesized, entrapped in liposomes and assessed in PDT against M6 and Q3Dau cultured human melanoma cell lines. Cell viability was determined spectrophotometrically by enzymatic clivage of the tetrazolium salt WST-1. After a-1 h compound incubation followed by 12 J per cm^2 light dose, the LD_{50} observed for Q3 Dau, similarly to M6 cells, were: 10^{-8} M (HexSiPc), 2×10^{-8} M (OctoSiPc), 10^{-8} M (CholoSiPc) and 2×10^{-8} M (CholSiPc). Compared to 10^{-6} M LD_{50} for dichloro SiPc (Cl_2SiPc), not bearing axial substituents, the gain in photocytotoxicity is two orders of magnitude. Moreover the Hematoporphyrin derivative (HpD) leads to only 5% cell killing at the same concentration. CholoSiPc administrated *in vivo* in PDT (0.1 μM per kg; 400 J per cm^2 visible light irradiation, 36 h after) of growing tumors from Q3Dau cells injected to nude mice, led to neoplasm necrosis. Taken together these results demonstrate that these new synthesized SiPc axially linked with cholesterol moieties or lipophilic chains exert a potent PDT killing effect on melanoma cells both *in vitro* and *in vivo* with advantages upon other known photosensitizers such as HpD, the purified fraction of Photofrin.

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Time and Dose Response Study of Topical PUVA Therapy: Cream-PUVA Therapy *Versus* Gel-PUVA Therapy

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Cream and gel PUVA are novel variations of topical PUVA therapy with good clinical efficacy. Studies on time and dose dependant phototoxic effects of these therapies are still lacking. We sought to assess the phototoxicity of both 8-MOP containing cream and gel preparations.

Thirty healthy volunteers (15 female, 15 males; skin type 2 and 3) were included into the study. The psoralen-cream (0.0006% 8-MOP, 0.001%, 0.0025% 8.MOP in three different vehicles) and gel (0.001%, 0.0025% and 0.005% 8-MOP hydrocellulose gel) preparations were assessed in a random plan. Application of 8-MOP-cream and -gel was directly followed by UVA administration (0.5–3.0 J per cm^2). Cream preparations were applied for 10, 20, 30 and 60 min. Gel preparations remained for only 15 min, as previously described. Erythema was assessed visually at 24, 48, 72 and 96 h after irradiation. The MPD, defined as the smallest dose of UVA to result in a just-detectable erythema, was determined 48, 72 and 96 h after UVA irradiation.

Twenty-four hours after application of 0.005%, 0.0025% and 0.001% 8-MOP-gel preparations severe erythema was detectable in all irradiated areas with a maximum after 96 or 108 h, in some volunteers accompanied by development of blisters. In those volunteers receiving 0.0006%, 0.001%, 0.0025 8-MOP cream preparations slight to distinct erythemas with a maximum peak at 72–96 h were observed. Only slight erythemas were seen after 10 min application of 8-MOP cream, while after 20, 30 and 60 min distinct eryhemas were observed. Maximal erythema after cream PUVA therapy was observed in all volunteers after 30 min. Phototoxicity of cream PUVA showed significant differences in dependence of the vehicle, in which 8-MOP was applied.

Our data give evidence that 8-MOP containing cream and gel preparations induce moderate to severe erythemas after topical application and irradiation with UVA. Phototoxicity of cream and gel preparations shows a great variety depending both on he vehicle and the time of application. These findings form the basis for he standardisation of clinical studies to determine therapeutic efficacy of topical PUVA therapies.

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Exposure to Various Ultraviolet Radiations in Human Skin *In Situ*: Biomarkers for Oxidative Stress

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Following UV radiation, reactive oxygen species (ROS) are responsible for the generation of oxidative stress in human skin. ROS are considered to play an important role in developing damage such as photoaging, immune suppression, cataract formation and carcinogenesis. The putative antioxidant defense system which protects human skin cells against developing such damage consists of two different types of protective molecules (enzymatic and nonenzymatic pathways). In this manner, we investigated the behavior of two enzymatic radical scavengers, superoxide dismutase (SOD) and heme oxygenase I (HO-1), in addition to heat shock protein 70 (Hsp 70) and ferritin following acute irradiation with UVA I, UVA I + II, and solar simulating light. Analysis of the content of these biomarkers in human skin after acute irradiation was performed by immunohistochemistry on biopsies from previously non-sun exposed sites of individuals with skin type II-III. In general we have found that their induction or depletion seems to be dependent on cell type, wavelength specificity and UV dose. The distribution of the first three of these proteins showed a uniform presence throughout the epidermis and ferritin was restricted to the basal keratinocytes of each volunteer before irradiation although heterogeneity was marked. Following acute UVA I (340–400 nm) irradiation with a dose of 1 or 2 MED a considerable dose-dependent decrease in antibody staining intensity was seen for SOD, HO-1, and Hsp 70 whereas ferritin was shown to increase or diffuse into supra-basal keratinocytes. The same responses concerning the content of SOD, Hsp 70 and ferritin occurred, but to a lesser extent, with UVA I + II and solar simulating radiation, whereas HO-1 was nearly unaffected by these two wavebands. It is obvious that the pro-antioxidant balance can be overwhelmed by acute photo-oxidative stress and this is of importance to determine how these reactions can be avoided in order to provide protection against subsequent oxidative stress.

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Quantification of UV-Induced DNA Damage by Laser Scanning Cytometry (LSC)

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Comet assay (single-cell DNA gel electrophoresis) is widely used in photobiology and toxicology to determine the degree of DNA damage induced by ultraviolet (UV) radiation or other agents. In principle, DNA fragments produced due to double-strand nicking by various mutagens or indirectly during DNA repair, are separated by electrophoresis from intact DNA. During single-cell electrophoresis the DNA fragments form a "tail" originating from cell nucleus, producing a comet-like figure. Usually, the amount of DNA in the tail is measured by image analysis and employed to quantify the degree of DNA damage.

We propose here an alternative method for DNA damage quantification that is based on measuring of DNA loss from the nucleus. The cells (HaCaT line or human peripheral lymphocytes) are prepared and electrophoresed on microscope slides, as in the classic comet assay. DNA is stained with propidium iodie, and fluorescence is measured by laser scanning cytometry (LSC) directly on microscope slides. In control, untreated cells a normal DNA histogram with low CV (<5%) was obtained with easily discernible G0/G1, S, and G2/M subpopulations. After induction of DNA damage by UVA radiation, a sub-G1 peak appeared on the DNA histogram. By re-scanning and digital imaging of the cells in the sub-G1 fraction we confirmed that they represented nuclei containing damaged DNA ("comets"). The fluorescence integral of the cells in the sub-G1 fraction correlated inversely with the tail moment (a measure of DNA content in the tail). Thus, LSC is a method for objective and fully automated DNA damage analysis. LSC enables precise quantification of the degree of damage, the proportion of damaged cells and correlating the degree of damage with the position in cell cycle.

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Sunscreens with High SPF Values are not Equivalent in the Protection From UVA Induced Polymorphous Light Eruption

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Polymorphous light eruption (PLE) represents an abnormal response of human skin to ultraviolet (UV) radiations, which is characterized by increased and prolonged expression of proinflammatory molecules such as ICAM-1 on the surface of keratinocytes (KC). Photoprovocation studies have previously revealed that in vast majority of PLE patients (80%) skin lesions can be induced through repetitive exposure to longwave UVA radiation (UVA 1, 340–400 nm).

In the present study we have assessed whether the generation of PLE lesions can be effectively prevented through pretreatment of human skin with three different sunscreen formulations having the following protection factors:

Sunscreen A: SPF > 75; UVA-PF 15 (IPD: Imediat Pigment Darkening method)
Sunscreen B: SPF = 35; UVA-PF (not known)
Sunscreen C: SPF > 60; UVA-PF 28 (PPD: Persistent Pigment Darkening method)

In this double blind, intraindividual comparative study, nine patients with UVA PLE were photoprovocated by exposing four sensitive skin areas to 100 J per cm^2 of UVA 1 on three consecutive days. Prior to photoprovocation, skin areas were either left untreated, or pretreated with cream A, B or C.

We have found that cream C was highly effective in providing complete protection against UVA radiation-induced skin lesions in nine of nine patients. In contrast cream A provided partial protection in seven of nine and complete protection in only two of nine, and cream B protected partially in one of nine and completely in none of nine patients, whereas eight of nine showed no protection.

The very high protective effect of cream C was corroborated by immunohistochemical studies in which strong KC ICAM-1 expression was found in unprotected areas, but not in cream C pretreated areas.

These studies clearly show that formulations having similar SPF values are not equivalent in preventing from UVA-induced PLE and that there is a need for products covering the entire UV spectrum, UVB + UVA. Moreover, we, for the first time, demonstrate the efficacy of a novel UVA filter (Mexoryl XL)

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A New Method to Determine UV-Penetration of Human Skin

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There is accumulating evidence that UV-radiation penetrating deep into the human dermis can promote carcinogenesis, collagen degradation and vascular cell adhesion. While for all these effects not only a dose dependence but also an exclusively relevant dose range has been identified *in vitro* there has been an astounding vagueness on actual dose ranges reaching the dermal structures *in vivo*. Therefore a new approach was developed facilitating exact measurements of tissue thickness and corresponding UV-penetrations using various preparations of human skin grafts *ex vivo* and a modified spectrophotometric apparatus. Thus compilations of UV-doses reaching the human dermis dependent on wavelengths could be generated ranging from 8 to 40%. Additionally, formerly neglected effects of tissue hydration could be clearly demonstrated: UV-penetration could be diminished down to 25% by hydration in normal saline corresponding to a 334% increase in tissue weight. Likewise, tissue exsiccation (air dried) caused a 16% increase of UV-transmission and a 20% decrease of tissue weight. These data provide reliable dose-ranges for UV-radiation reaching vital dermal structures which had not been established previously. More over the methodology opens new possibilities to validate the effectiveness of sun protection formulas in shielding dermal structures.

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Differences in CD11b+ Cell Population and Apoptosis Between Patients with Polymorphous Light Eruption and Healthy Individuals

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After UVB irradiation Langerhans cells disappear from the epidermis. Subsequently CD11b+ macrophagic cells, which are reported to produce the immunosuppressive cytokine IL-10, infiltrate the epidermis of healthy individuals. The overall effect of UVB on healthy skin is immunosuppression. A disturbance of the immunosuppressive mechanism might lead to an adverse immune reaction, i.e., like in polymorphous light eruption (PLE).

We found recently that the CD1a+ Langerhans cells in the UVB overexposed buttock skin of PLE patients did not disappear after 6 MED overexposure, in contrast to the Langerhans cells in the UVB exposed skin of healthy volunteers. Furthermore, the CD11b+ macrophagic cells that increased in the dermis and infiltrated the epidermis of healthy individuals after UVB irradiation, hardly infiltrated the epidermis of PLE patients.

To characterize the CD11b+ cells further we performed immunohistochemical staining with several markers (CD68, elastase). It appeared that the CD11b+ cells that infiltrated the epidermis in healthy individuals after UVB overexposure were CD68– (pan-macrophage marker), whereas the few cells that infiltrated the epidermis of PLE patients were all CD68+. The dermal CD11b+ cells in the unexposed buttock skin of PLE patients and healthy volunteers were almost all CD11b/CD68 double positive. The dermal CD11b+ cells in the UV-exposed buttock skin of healthy volunteers were only partially CD68+, while in the UV-exposed skin of PLE patients the CD11b+ cells were almost all CD68+. Double staining of the CD11b + cells with elastase revealed that the CD11b single positive cell that infiltrated the epidermis of healthy individuals but not PLE patients after UV irradiation were all elastase+ and therefore neutrophils. Because the Langerhans cells in PLE patients seem to be UVB resistent, we speculated that this might be caused by a disturbance in apoptotic response. Preliminary results show that apoptosis (detected via antiactive caspase 3) is present in the UV-exposed skin of PLE patients but no double staining with the Langerhans cell marker CD1a has been observed thus far.

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Zinc Protects Genomic DNA from Solar Light Injury: A New Role for Zinc Induced Metallothionein in Photoprotection?

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Zinc (Zn) protected cells from UV-induced cell death both by apoptosis and necrosis. It has been proposed that metallothionein (MT), a rich cystein protein, may be a good candidate to explain this cytoprotective effects. Indeed MT acts as a free radical scavenger and as a Zn donor for Zn-metalloenzymes. In this work we investigated the role of Zn-induced MT in the protection of genomic DNA from solar simulated light. Human keratinocyte cells (HaCat) were treated with zinc chloride (100 μM) for 24–72 h. DNA strand-breaks and alkali-labile sites were measured using the comet assay. Solar-simulated light (1.5 J per cm^2 corresponding to 1 minimal erythemal dose) induced DNA damage. Zn significantly protected genomic DNA and. this protection is time dependent. MT was investigated by immunostaining or western blotting and its localisation confirmed with ^{75}Zn labelling. In Zn-treated cells a new repartition for MT was observed and a nuclear pool can then be visualised. In irradiated cells, the same pattern was observed. The influence of Zn treatment on this expression was investigated. It has been reported that irradiation induced glutathione (GSH) depletion. Therefore we studied the influence of a chemical GSH depletion on MT synthesis. In GSH depleted cells an induction of apoMT was visualized. These results confirm the link between MT and GSH. In contrast, in Zn treated cells GSH depletion decreased MT expression.

In conclusion Zn-MT could maintain cellular redox potential and partially explain the protective effects of Zn against DNA damage. Nevertheless the consequence of apoMT induction by a single solar light irradiation in Zn-deficient cells remained to be elucidated.

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Prevention of Solar Induced Immunosuppression by a New Highly Protective Broadspectrum Sunscreen

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It is now well established that ultraviolet radiations are responsible for alterations of the cutaneous immune system and may be at the ethiology of skin cancers. Recently, it has been clearly demonstrated that not only UVB (290–320 nm) but also UVA (320–400 nm) can be responsible of these effects. Thus, sunscreen products highly protective in the UVB range (erythema) are less effective in preventing the UV induced changes in the skin immune function than those covering the entire UVB + UVA spectrum.

We previously studied in human the effect of exposure to either UVB + UVA or only UVA on the delayed-type hypersensitivity response (DTH). DTH was assessed using a Multitest kit (Pasteur/Mérieux), providing an original approach to evaluate modifications in the cutaneous immune capacities.

The aim of the present work was to evaluate, in humans volunteers, under real sun exposure conditions, the efficacy of a new broadspectrum sunscreen product in preventing loss of DTH response.

DTH tests were performed before and after sun exposure of the upper part of the back. A non exposed area (forearm) was used as control. Prior to sun exposures 14 subjects of were treated with the new sunscreen formula. This sunscreen has both a very high sun (SPF > 60) and UVA (UVAPF = 28, Persistent Pigment Darkening method (PPD)) protection factors. Moreover, the following UV filtering system: UVB filter (Octocrylene), UVA filters (Mexoryl SX, Mexoryl XL, Parsol 1789), TiO^2 allows the formula to be photostable.

The volunteers were sun exposed during 6 d. They received a total UV dose equivalent to 64 MED and 400 J per cm^2 of UVA. Compared to the DTH response we obtained before sun exposure, we did not detect any changes in the immune response when skin was protected by the sunscreen formula.

We have demonstrated that, under intensive sunlight exposure, the use of a highly protective UVB + UVA sunscreen can prevent from photo-immunosuppression. This is of particular importance if we consider the possible link between immunosuppression and skin cancers developments.

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The Local and Systemic Response of Human Epidermal Langerhans Cells Towards Artificial UV Irradiation *In Vivo* is Not Altered by Oral β-Carotene Substitution

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Beta-carotene (BC), a radical scavenger with pro-vitamin-A-activity is used in clinical dermatology to prevent UV-induced skin damage but the mechanisms of BC action on the skin immune system are still not well understood. Since prior studies had shown a preventive effect on erythema development and DTH reaction, we investigated the effects of an 8-wk oral BC supplementation (30 mg per d, 15 mg per d, 5 mg per d, placebo) on epidermal Langerhans cells response to a 14-d suberythrogenic UV irradiation. Following supplementation, 68 healthy female volunteers were shave biopsied before irradiation, whole body irradiated daily for 2 wk and then biopsied from irradiated and nonirradiated control skin sites. Epidermal cell (EC) suspensions were prepared and Langerhans cell numbers and surface molecules were analyzed by immunostaining and flow cytometry. Irradiation with UVA/UVB led to a highly significant ($p = 0.0001$) decrease of Langerhans cells in the EC suspension of an irradiated area, UVA alone had similar ($p = 0.078$) effects. BC substitution showed a dose independent trend towards depletion protection. In 15% of the UVA treated and 65% of the UVA/UVB treated volunteers, a CD36, CD1a and CD11b positive cell population was observed in the irradiated skin irrespective of BC substitution. Following two weeks of daily UVA/UVB, but not UVA exposure, a significant upregulation of FcγRI/CD32 on Langerhans cells from irradiated and nonirradiated skin areas was seen, suggesting a systemic UVB-mediated effect. All other surface receptors investigated did not show evidence of UV-dependent or BC responsive regulation. In conclusion, the investigated local and systemic alterations of the skin immune system induced by suberythrogenic, artificial UV irradiation are unresponsive to oral BC substitution, emphasizing that use of an effective sunscreen should not be substituted by an oral BC regimen alone.

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Antioxidant Status and UV Sensitivity of Human Keratinocytes Harboring Human Papillomavirus Type 16

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SKv keratinocyte lines harboring human papillomavirus type 16 (HPV16) were established from a vulvar bowenoid papulosis lesion (1). These cell lines displayed different autoregulation by tumor necrosis factor-α and different. *in vitro* proliferative potential which correlated with tumorigenicity in mice (2). Immortalization and malignant transformation of "high risk" HPV-infected cells are dependent on the expression of two oncoproteins E6 and E7. The aim of our study was first to evaluate the respective antioxidant capacities of weakly (SKv-e) and high (SKv-l) tumorigenic SKv cell lines and to determine their sensitivity to solar simulated radiation. We found significant higher level of glutathione and glutathione peroxidase activity in SKv-l compared to SKv-e (respectively threefold and fourfold). No significant difference was found concerning superoxide dismutase activities. Interestingly, SKv-e and SKv-l showed significant different sensitivity when exposed to a single solar simulated irradiation. The lethal dose 50, determined by MTT test, corresponded to 0.3 MED for SKv-e and 1 MED for SKv-l (MED = minimal erythemal dose evaluated to 1.5 J per cm^2). In the second part of our study, we investigated the expression of cell cycle proteins (p53, p21) in these HPV16 keratinocyte cell lines in basal conditions and after a single solar simulated irradiation.

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A New Option for Treating Alopecia Areata: PUVA-Turban

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Amongst multiple systemic as well as topical treatment modalities, orally administered PUVA therapy has been reported as a therapeutic alternative in alopecia areata. The clinical use of oral PUVA, however, is often limited by systemic side-effects. A solution of this problem is offered by bath PUVA-therapy due to a near complete lack of systemic resorption of psoralen. Through use of a "PUVA-turban" it is possible to administer a dilute bathwater solution containing 8-methoxalen (8-MOP) to the scalp.

We wanted to clarify if "PUVA-turban-therapy" may be effective in treating alopecia areata in different clinical stages.

We treated 10 patients with severe, rapidly progressing alopecia areata, which had been resistent to prior local and systemic therapy, with PUVA-turban-therapy as modified bath PUVA-therapy. Each treatment session two cotton towels were soaked with a psoralen-containing solution at 37°C (8-MOP-concentration: 1 mg per liter), wringed gently to remove excess water and wrapped around the patient's head in a turban fashion for 20 min. This was directly followed by UVA radiation. Treatment sessions were initially performed three to four times per week. The cumulative UVA doses given over treatment periods of up to 24 wk were 60.9–188.2 J per cm^2 with single doses ranging from 0.3 to 8.0 J per cm^2. After up to 10 wk of treatment hair regrowth could be noticed in seven of 10 patients. Two patients did not respond to our treatment, one patient showed only incomplete hair regrowth.

PUVA-turban-therapy as a variant of PUVA-bath-therapy can be considered a useful method of administering dilute psoralen solution selectively to the scalp of patients. It has shown to be a well tolerated and, in some patients, efficient therapeutic alternative in the treatment of alopecia areata.

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PUVA-Bath Photochemotherapy in Patients with Chronic Sclerodermic Graft *Versus* Host Disease

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Sclerodermic graft *versus* host disease (S-GvHD) is a severe cutaneous complication of allogenic bone marrow transplantation occurring in up to 10% of patients with GvHD. Conventional immunosuppressive treatment modalities often could not halt progression of disease and soften sclerodermic skin lesions.

We here report five patients suffering from S-GvHD with joint contractures who did not respond to a combination therapy of cyclosporine A plus prednisone and/or mycophenolate mofetil (MMF) plus prednisone. In all patients, PUVA-bath photochemotherapy was initialized four times a week using a standardized treatment protocol and predefined UVA doses. In addition to PUVA-bath photochemotherapy treatment with MMF was continued while therapy with other immunosuppressive drugs was stopped.

In all patients, skin lesions improved after 12–16 treatment sessions, and MMF could be reduced after 20 irradiations. Sclerodermic lesions softened markedly after 22–25 treatment sessions using a clinical scoring system. 20 MHz ultrasound evaluation showed features of normal skin concerning skin thickness and density after 25 treatments. Follow up over up to 6 mo showed no relapse or worsening of the skin conditions.

Our findings strongly suggest PUVA-bath photochemotherapy to be a very effective treatment for S-GvHD. PUVA-bath photochemotherapy using a standardized treatment protocol with predefined UVA doses may result in striking clinical improvement, even in patients with reduced joint mobility and skin changes refractory to immunosuppressive treatment before. Further controlled studies are necessary to confirm these observations.

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Successful Remission of Acute Graft-*Versus*-Host Disease After Extracorporeal Photoimmunotherapy (ECP)

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Extracorporeal exposure of peripheral blood leukocytes to the photosensitizing compound 8-methoxypsoralen (8-MOP) and ultraviolet A (UVA) radiation is a effective therapy for selected T-cell mediated diseases including rejection after organ transplantation (Heart, lung). Even though more patients with chronic graft-*versus*-host disease (GvHD) have been reported to respond to ECP, only anecdotal reports on patients with acute GvHD and ECP exist. We present 17 patients (seven male, 10 female) with hematological malignancies with a median age of 38 (range, 27–49) y who received marrow graft from sibling (n = 2) or unrelated (n = 15) donors. Twelve to 30 d after bone marrow transplantation (BMT) they developed acute GvHD grade II (n = 7), III (n = 6) IV (n = 4) according to the IBMTR criteria. All patients received treatment with cyclosporine and steroids at 2–10 mg per kg and were refractory to the latter. After a median of 46 d (range, 30–75 d) after BMT, ECP was initiated and performed on two consecutive days every 1–2 wk for the first three months and thereafter every 2–4 wk until resolution of acute GvHD. A median of 13 (range 2–45) cycles of ECP were performed. Three patients died after 2–3 cycles of ECP due to infection associated with acute GvHD grade IV. Following ECP, 10 of 14 (71%) had complete resolution (CR) of GvHD, one of 14 partial resolution (PR) and three patients are currently still under treatment. Six patients developed chronic GvHD 5–28 mo after BMT, all other complete responders had sustained clinical and hematologic resolution of GvHD activity. Except for a reversible fall in peripheral blood cell counts in some patients, no significant side-effects under ECP were observed. Our findings suggest the concept that ECP is a safe and effective adjuvant therapy for acute GvHD without severe gastrointestinal involvement and resistance to standard immunosuppressive therapy.

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Absence of Correlation Between Erythema and DNA Damage after UV Irradiation of Human Skin

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The use of high sun protection (SPF) sunscreens is proposed to reduce skin cancer incidence. Relationship between erythema and DNA damage is however, a matter of debate. The objective of this study is in volunteers irradiated with a biologically similar dose of 2 minimal erythema doses (MED) to quantify the intensity of UV-induced DNA damage via DNA repair or unscheduled DNA synthesis (UDS) and via the density of apoptotic cell death or sunburn cells (SBC).

Fifteen human volunteers (phototype II or III) were irradiated by a solar simulator at a dose of two MED's at two different sites. The first site was biopsied after 15 min, incubated in 3H-thymidine and submitted to autoradiography for UDS determination. The second site was biopsied 24 h later for SBC density.

UDS and SBC are not correlated with erythema reaction as measured by the MED. In contrast, SBC density grows with the intensity of UDS confirming that the two phenomenon are biologically related. These results suggests that the cancer protection offered by the sunscreens is limited and not related to the SPF.

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Induction of DNA Strand Breaks by 8-Methoxy-Psoralen and UVA (PUVA) in Cultured Cells Detected by Means of Comet-Assay

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The DNA is one of the essential UV-targets within the cell. The aim of the present study was to investigate DNA-damage and -repair following the treatment of HaCaT-cells by PUVA in comparison with the effects of UVB and UVA-irradiation. Comet-assay (single-cell gel electrophoresis) was used to evaluate DNA strand breaks and alkali labile sites caused by UV. Our results suggest that the mechanism of the effects of UVB and UVA for the formation and repair of DNA-damage is quite different from that of PUVA. Immediately after irradiation comet-formation by alkaline assay was found only in the case of UVA (0–5 J per cm^2). After 1 h the length and intensity of the comet were decreased because of the fast repair of DNA single strand breaks caused by UVA. After UVB (0–60 mJ per cm^2) the comet-formation was detected only 1 h after irradiation. This comet can be the result of DNA single strand breaks caused by nucleotide excision repair mechanism. Neither UVA nor UVB caused DNA-damage detectable by neutral comet-assay. It means that UVA and UVB can not induce DNA double strand breaks within the dose ranges studied. After PUVA-treatment (300 ng per ml 8-MOP and 2 J per cm^2 UVA) we observed increasing comet-formation with a peak of 1.5 h after irradiation by neutral assay, then the comet-tails decreased slowly during the next few hours. It suggests the occurrence of DNA double strand breaks as secondary DNA-lesions. Presumably they are repair intermediary products of the DNA-cross links caused by PUVA.

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Cream-pUVA Therapy for Treatment of Scleroderma Adultorum Buschke

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Numerous treatments have been tried for scleredema adultorum (SA), a rare disorder of unknown cause, but none have proved to be effective. Recently, three cases of successful treatment of SA with PUVA-bath therapy have been described. However PUVA bath therapy may be difficult to manage as it requires bath tubes. An elegant new variation of topical PUVA therapy is cream PUVA with 8-MOP containing cream preparations. We decided to evaluate the effect 0.001% 8-MOP cream in one patient with SA.

A 55-y-old man (skin type 3), suffering from non insulin dependant diabetes mellitus, had a 10-y history of progressive tightness of the skin of his back. Before treatment, diagnosis was confirmed by histopathologic analysis and 10 MHz ultrasound assessment of representative sclerotic areas. Intravenous penicillin remained uneffective. After determination of the patients minimal phototoxic dose we started cream PUVA therapy with 0.001% 8-MOP cream. 8-MOP-cream was applied for 60 min in an even layer on the back and directly followed by UVA administration with increasing doses (initial dose 0.3 J per cm^2) up to a maximum dose of 5.0 J per cm^2. Total number of treatments was 30, total cumulative UVA dose was 91 J per cm^2.

Comparison of ultrasonographic pictures before and after therapy showed a significant reduction of both sonographic density and thickness of the involved skin. A biopsy specimen taken before treatment showed marked thickening of the dermis with thickened collagen bundles separated by clear spaces with mucin lying between them. A postbiopsy specimen revealed almost normal skin. Our observation provides evidence that cream PUVA therapy may be also successful for the treatment of SA. The mechanism responsible for the reduction of skin thickness in SA with topical PUVA therapy is unknown but it is possible that it may modulate the biosynthetic capacities of dermal fibroblasts. The clinical effectiveness of cream PUVA in this patient makes this novel therapeutic option a promising tool to be evaluated in future studies.

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Psoralen plus Ultraviolet A Light Arrests Keratinocytes at the G2/M Boarder of the Cell Division Cycle

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8-Methoxypsoralen (8-MOP) plus ultraviolet A light (PUVA) is used for the treatment of a range of hyperproliferative and inflammatory skin conditions. PUVA causes damage to cellular DNA by the formation of 8-MOP/DNA adducts. To ensure the integrity of the genome, cells arrest in response to DNA damage at certain checkpoints during their division cycle.

In order to investigate the effect of PUVA on the cell division cycle, HaCaT keratinocytes were treated with increasing doses of PUVA. 24 and 48 h thereafter, cells were stained with propidium iodide and analysed for DNA content by flow cytometry. Doses ranging from 0.1 μg per ml 8-MOP plus 0.05 J per cm^2 UVA to 0.1 μg per ml 8-MOP plus 0.1 J per cm^2 UVA caused HaCaT keratinocytes to accumulate in the G2 phase of the division cycle, indicating that PUVA activates the G2/M DNA-damage checkpoint in HaCaT cells. At higher doses of PUVA, cell cycle progression was blocked nonspecifically at all stages of the division cycle, presumably due to severe impairment of more general cellular functions.

Our findings suggest that activation of cell cycle checkpoints plays an important role in the antiproliferative effect of PUVA therapy.

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UVB Light Suppresses, and All-Trans-Retinoic Acid Induces Apoptosis in Human Squamous Cell Carcinoma Cell Line Scc-12, Both Together Induce Apoptosis Synergistically

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*Objective:*The impact of UVB light and retinoids on the induction of the programmed cell death (apoptosis) in epidermal malignancies is barely understood. Therefore we addressed the question whether *all-trans*-retinoic acid (atRA) influences the UV-B induced apoptosis in SCC-12 cell line (SCC-12), and whether UV-irradiation has any influence on the metabolism and isomerization of retinoids in skin cells.

Methods: Confluent SCC-12 were radiated with UV-B light (0.05–20 mJ per cm^2), consecutively treated with atRA 10^{-5} M and incubated for 96 h in the dark. Then, total-DNA was extracted, and apoptosis was determined by apoptotic-ladder-PCR. Simultaneously, the metabolism and isomerization of retinoids was determined from cell extracts by RP HPLC.

Results: As revealed by "Apoptotic-Ladder-PCR" UV-B light (12 mJ per cm^2) decreased the apoptosis rate as compared to nonirradiated SCC-12 significantly. The addition of atRA after UVB radiation significantly induced apoptosis as compared to atRA treatment of nonradiated cells, and UVB exposed cells without atRA-treatment. Simultaneously, increased levels of *9,13-dicis*-RA and a decrease of atRA were measured by RP-HPLC in UVB irradiated cells.

Conclusion: The maximal induction of apoptosis in atRA-treated SCC-12 after UVB radiation may suggest that atRA is able to restore the ability of SCC-12 to react to UVB distress by inducing the apoptosis. This is remarkable since UVB alone suppresses apoptosis in this cell line, which suggests that SCC-12 are not able to react adequately to UVB-distress and related cell damage. The increased synthesis of the storage-isomer of atRA, *9,13-dicis*-RA, after UVB-radiation suggests that the intracellular homeostasis of retinoids is disturbed by UV-light dose dependently. To what extent the expression of retinoid receptors is of relevance here, is a current topic of investigation in our laboratory.

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UVB-Irradiation Suppresses the Basal Expression and Inducibility of the *all-trans*-4-Hydroxylase (CYP26) in HaCaT Cells

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The cytochrome P450 multigene family of heme proteins catalyze the oxidation of various xenobiotics and endogenous molecules. One member of this family is the *all-trans* retinoic acid-4-hydroxylase (Cytochrome 26, CYP26) which catalyzes the 4-hydroxylation of *all-trans*-retinoic acid. The expression of the CYP26 in human skin is essential for the cleavage of the hormone active retinoid *all-trans* retinoic acid. In previous studies we have shown that this enzyme is inducible in human dermal fibroblasts and human epidermal squamous cell carcinoma cell line SCC-12. In the present study the effect of UV-B exposure on the CYP26 activity in human epidermal HaCat cells was determined on the RNA-level by semiquantitative rt-PCR, and on the protein level by reverse phase high performance liquid chromatography (RP-HPLC).

RP-HPLC analysis of UV-B-irradiated cells (0,1–1–6–12–20 mJ per cm^2) shows a dose dependent decrease of 4-hydroxy-metabolites of *all-trans* retinoic acid while the intracellular levels of *all-trans* retinoic acid remain stable. Semiquantitative rt-PCR reveals an abolished basal expression, and a decreased inducibility of the CYP26 mRNA expression, while the expression of the beta actin gene (internal control) remains constant.

These results demonstrate that UV-B-irradiation of skin cells can alter the metabolism of retinoids in a dose dependent fashion. To what extent this modulation affects cell proliferation, differentiation, and the regulation of apoptotic processes in other dermal and epidermal cells is currently under investigation in our laboratory.

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Expression and Activation of Pro-Gelatinase A by Human Melanoma Cell Lines with Different Tumorigenic Potential

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The production of various proteolytic enzymes by tumor cells is thought to facilitate the invasion of solid tumors into surrounding tissues. We examined three cell lines (M1Dor, M4Be and M3Da) derived from malignant melanoma for which previous studies demonstrated their different abilities to grow in nude mice after subcutaneous grafting. By invasion assay *in vitro* using Boyden-chambers technique, we found that none of those cell lines were able to invade the Matrigel. Several studies have substantiated the role of matrix metalloproteinases, mainly gelatinases MMP-9 and MMP-2, in melanoma cell invasion. We found that each cell line constitutively produced MMP-2 in its latent form (72 kDa) only, with stronger production for the most invasive cell line *in vivo* (M3Da). Integrity of the MMP-2 activation process was studied to relate the *in vivo* invasive potential to MMP-2 production since MMP-2 was recovered in its inactive form at cell plasma membrane. TIMP-2 and MT1-MMP were secreted in a constitutive manner by the three cell lines but inversely related to the cell tumorigenic potential. The TIMP-2/MMP-2 secreted ratio was calculated for each cell line that showed a large excess of TIMP-2 (M1Dor: 334, M4Be: 100, M3Da: 10) which may explain the absence of pro MMP-2 activation. Furthermore, high levels of membrane-bound TIMP-2 were found which might saturate all the MT1-MMP molecules on the cell surface, leaving no free MT1-MMP available to cleave progelatinase A bound in the triplex with TIMP-2 and MT1-MMP. Plating cells onto type I or type IV collagen did not trigger pro-MMP-2 activation. On the contrary, conversion of pro-MMP-2 to its active species could be evidenced when melanoma cell lines were seeded in a three dimensional type I collagen lattice. Those data gave further insight on the importance of the type I collagen organization around cancer cells to direct proteolysis maybe *via* a MMP-2 intracellular activation process.

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Butyric Acid Effects on Melanoma Cell Lines: Apoptosis Induction and Cell Cycle Cytofluorimetric Evaluation

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Butyric acid is a non toxic natural product found in food and present in the digestive system as a bioproduct of microbial fermentation; it is able to induce a series of cellular alterations (disregulation of cell cycle and induction of apoptosis) in different tumour cell lines (human colorectal, lymphoid, and breast cancers). However, few data are available on melanoma cell lines. This prompted us to investigate the effects exerted by butyric acid on the cell growth and cycle perturbation of one murine melanoma cell line (B16) and one human melanoma cell line (HMCL) derived from a subcutaneous metastasis in our laboratory. The two cell lines were allowed to attach and then the culture medium was supplemented with increasing concentrations (0.1–0.5–1.0–3.0 mM) of butyric acid (Sigma) vehiculated by solid lipid nanospheres. Cell cycle perturbation, apoptotic cell percentage and cell vitality was analysed 1, 2 and 3 d after butyric acid treatment. The antiproliferative and apoptotic effects were evaluated in terms of DNA content variation as determined by propidium iodide and propidium iodide/annexin V staining. Vitality was evaluated by trypan blue incorporation. Flow cytometric analyses evidenced a $G_0/_1$-S transition block and a sub-G_1 apoptotic peak starting from 0.5 to 3.0 mM butyric acid. The percentage of apoptotic cells did not change throughout all the experiment time in untreated cells (mean 9% in B16 and 12% in HMCL). After a 24-h-butyric acid treatment, a dramatic dose dependent increase in the apoptotic cell percentage was found in both cell lines (27% at 0.1 mM, 85% at 0.5 mM, 90% at 1.0 and 3.0 mM in B16; 23% at 0.1 mM, 75% at 0.5 mM, 89% at 1.0 and 3.0 mM in HMCL). On the other hand no significant differences were observed between 24 h- and 48- or 72 h apoptotic cell percentage. Similarly, a reduced B16 and HMCL vitality was found after butyric acid treatment, from a mean of 94% on untreated cells to 13% at 0.5 mM and 0.5% at 1.0–3.0 mM. Taken together, our results show that butyric acid exerts a dose-dependent but not time-dependent effects in melanoma cell apoptosis induction.

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Detection of Melanoma Cells in Blood

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The detection of circulating melanoma cells by molecular biological methods has been the object of recent investigations. However, published results are contradicting; detection rates vary from 0% to 100%. We present a new, cellular approach to identify circulating melanoma cells in peripheral blood using immunomagnetic cell sorting.

178 blood samples from 129 melanoma patients and 30 samples from healthy persons and nonmelanoma patients were examined. After density gradient centrifugation, the interphase was incubated with the murine mAb 9.2.27. The antibody recognizes a human melanoma-associated chondroitin sulfate proteoglycan, which is expressed on more than 90% of all melanoma cell lines examined. 9.2.27 positive cells were labeled with magnetic microbeads and enriched by immunomagnetic cell sorting. Cells were stained using an alkaline phosphatase-anti alkaline phosphatase assay and examined by light microscopy.

In spiking experiments, melanoma cells, seeded at a concentration of one melanoma cell per ml whole blood, could be detected reliably with the assay. Circulating melanoma cells were not found in 30 controls examined, nor were 9.2.27-positive cells found in 41 patients with primary malignant melanoma. In patients with regional lymph node metastases, circulating 9.2.27-positive cells could be detected in three out of 22 patients (13.6%). All three positive patients presented with disseminated metastases 4–8 wk after surgical removal of their metastases. In patients with disseminated disease, circulating melanoma cells were found in 10 out of 66 patients (15.2%) examined. The number of positive cells varied from 2 to 2364. This corresponds to about 0.5 to more than 400 melanoma cells per ml whole blood.

We conclude that immunomagnetic cell sorting is a sensitive and specific approach to detect circulating melanoma cells in peripheral blood. Circulating melanoma cells could be found in a number of patients with advanced disease. The method is not suitable for early detection of metastases. However, a positive result may be indicative of bad prognosis. The main advantage in comparison to PCR techniques is, that not only mRNA sequences but whole melanoma cells may be identified. The method is a valuable tool to further investigate biological characteristics of circulating melanoma cells.

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Adhesive Properties of Melanocytes are Modified by UV-Irradiation

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The interaction of melanocytes with the extracellular matrix mediated by adhesion molecules is important for many biological processes like migration of melanocytes from neural crest, repigmentation, and melanocytic tumor progression. To investigate the ability of UV-light to influence the adhesion of melanocytes to fibronectin cultured melanocytes were exposed to increasing doses of UV-light (solar simulator, 10, 20, 30 mJ per cm^2). The number of adhered cells are determined by an enzyme based color reaction. To prove the role of fibronectin-specific integrins antibodies (ab) against integrins $\alpha5\beta1$ and $\alpha v\beta3$ were preincubated. A UV-dose-dependent increase of the attachment of melanocytes to fibronectin was observed. The UV-induced increase was suppressed in the inhibition assay with preincubation of specific ab. Immunohistochemically the staining signal after incubation of ab against $\alpha5\beta1$-and $\alpha v\beta3$-integrin was enhanced which was also confirmed by flow cytometric analysis. The findings clearly underline the capability of UV-light to alter the adhesion of melanocytes to fibronectin *in vitro*. Adhesion to laminin and collagen type IV was not influenced by UV in our assay. *In vivo*, the situation is far more complicate because other cells than melanocytes may be influenced by the UV-exposure. After UV-irradiation the integrin expression within the epidermis is altered. Therefore, both keratinocytes and melanocytes may be involved by modulation of the adhesive properties in processes like repigmentation of vitiligo skin during UV-therapy or morphologic changes reported to occur in melanocytic nevi after UV-exposure.

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Reconstructed Epidermis With and Without Low Phototype Melanocytes: Comparative Study of UVB, UVA and UVA + B Irradiation

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The reconstructed epidermis with melanocytes (REM) and without melanocytes (REK) has already been submitted to UVB irradiation to study photoprotection and pigmentation. To improve its potential for studying sunscreens or antioxidants, we compared effects of UVB, UVA and UVA + B. Reconstructs were made according to a modification of the Pruniéras technique and irradiated at 312 nm, or/and 365 nm using a Biotronic Vilber Lourmat device. Melanocytes originated from donors of phototypes II or III. Twenty-four hours following irradiation, we studied sunburn cells (SBC), protein oxidation and antioxidant enzymes (catalase, superoxide dismutase). We also studied apoptosis by TUNEL and NO production by nitrate and nitrite dosage. Oxidated proteins were detected without irradiation but their amount was increased after irradiation. SBC were more specifically UVB induced, whereas catalase and superoxide dismutase alteration were more specifically UVA induced. Protein oxidation due mainly to free radicals was provoked by UVA and UVB as well.

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Ganglioside-Based Epidemiology of Malignant Melanoma

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GD3, one of the major melanoma gangliosides, could be a key ganglioside in growth and proliferation of melanoma, because GD3-deficient mutant human melanoma cell lines exhibited lower proliferative response *in vitro*. The aim of the present study was to investigate the relationship between the prognosis of melanoma and polymorphisms of ganglioside in Japanese patients.

Ganglioside profiles of melanoma tumor tissues were performed by thin-layer chromatography. Eighteen patients were included in this study. Regarding the clinical subtypes of melanoma, nine were nodular melanoma (NM), seven acral lentiginous melanoma (ALM), and one superficial spreading melanoma and lentigo maligna melanoma, respectively.

An analysis of ganglioside profiles showed new type of expression, that is, GM3 was nearly the only ganglioside (>95%) in three patients with ALM (three of seven of ALM – 42.9%). In contrast, this new ganglioside profile was absent among the nine NM patients. Two metastatic melanoma patients (stage IV) with new type of ganglioside expression (GM3-predominant) seemed to survive longer than four patients (stage IV) with increased levels of both GM3 and GD3 (45 mo *vs* 8 mo; $p < 0.05$). Thus it way be noted that variations of ganglioside in melanoma could be useful in seeking out a new immunotherapy leading enzymatic modification associated with glycosylation.

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Influence of MIP-3α on the Migratory Capacity of Immature Dendritic Cells/Langerhans Cells

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If the stimuli that induce human Langerhans cells to leave the epidermis to the draining lymph nodes are rather well characterized, nothing is known concerning their traffic from blood to the epidermis. The *in vitro* controlled development of DC/Langerhans cells from CD34+ hematopoietic progenitor cells now allows to address this issue. The observation that MIP-3α may be involved in the attraction of Langerhans cells into the epidermis prompted us to study the effect of this chemokine on the ability of immature DC/Langerhans cells to migrate through a reconstituted basement membrane (Matrigel). Indeed, *in vivo*, to migrate from the blood to the epidermis immature DC must move through the dermal-epidermal basement membrane. DC were differentiated from cord blood CD34+ cells cultured for 6 d with GM-CSF plus TNF-α, in the presence or the absence of TGF-β1. Day 6 DC/Langerhans cell progenitors migrate in a dose dependent fashion in response to MIP-3α, from 31.5% (0.25 μg MIP-3α) to 48.3% (1 μg MIP-3α) in the absence of TGF-β1. MIP-3α preferentially attracts CD1a+ cells rather than CD14+ cells (40.5% *vs* 16.1%). Only 11.4% of the migrated cells express CD1a/E-cadherin molecules indicating they are Langerhans cells. 95% express the cutaneous lymphocyte antigen (CLA). In the presence of TGF-β1, up to 64% of cells migrate in response to 1 μg of MIP-3α, among them all the CD1a+ cells express CLA and 50,5% being CD1a+/E-cadherin display the phenotype of Langerhans cells. These cells further cultured until day 12 with GM-CSF, TNF-α and TGF-β1 preserved their phenotype of epidermal Langerhans cells. Our results show that a great number of immature DC, whatever their phenotype is, is able to migrate through Matrigel in response to MIP-3α. In the presence of TGF-β1, it appears that the number of DC/Langerhans cells which responds to MIP-3α is specifically increased. Our results underline the role of TGF-β1 in the migration of immature Langerhans cells into the epidermis.

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Expression of Nerve Growth Factor Receptors on Immunocompetent Cells

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Increasing evidence indicates that nerve growth factor (NGF) can act as a regulatory molecule during inflammatory and immune responses. In the present study, we investigated the expression of NGF receptors on human lymphocytes, monocytes and monocyte-derived dendritic cells (DCs). Resting B lymphocytes and various subsets of T lymphocytes ($CD4^+$, $CD8^+$, $CD45RA^+$ and $CD45RO^+$) isolated from peripheral blood did not express TrkA or the low affinity p75 receptor, as assessed by flow cytometry and RT-PCR analysis. Upon activation with lectins, both B cells and T cells upregulated selectively TrkA expression. Unstimulated $CD14^+$ monocytes expressed TrkA, but not p75 receptor, at both mRNA and protein levels, and using Ab recognizing extracellular and intracellular epitopes. On these cells, NGF or the TrkA agonist Ab, 5C3, was able to prevent apoptosis induced by gliotoxin. In contrast, TrkA stimulation did not affect MHC molecules, CD86, CD40 or CD14 expression, or the alloantigen presenting function of monocytes. During culture with GM-CSF and IL-4 and transition to DCs, expression of extracellular TrkA epitopes recognized by two different mAbs was progressively lost although the intracellular portion and specific mRNA were still present. Stimulation of DCs with LPS, LTA, TNF-α or CD40L did not change this pattern of expression. As a consequence of the lack of the extracellular TrkA domain, immature DCs were not sensitive to NGF in terms of cell survival, membrane phenotype, cytokine release or APC function. The results indicate that NGF can regulate lymphocyte and monocyte functions, but has no major effects on monocyte-derived DCs.

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Triggering CD101 Molecules Increases IL-10 Production While Inhibiting Cell Proliferation Induced by Skin Dendritic Cells

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Activation of T lymphocytes by antigen -presenting cells requires costimulatory signals in addition to the primary signal provided by the engagement of the T cell receptor. MHC class 1 and class 11 antigens, as well as counterreceptors to costimulatory and adhesion molecules have been identified on the surface of skin dendritic cells and are crucial for their function. CD101 molecule is a transmembrane protein containing seven immunoglobulin type lgV domains. We have previously shown that CD101 is expressed on a major subpopulation of HLA-DR+, CD1a+, CD1c+ skin dendritic cells, and on activated T cells. We further studied the functional role of CD101. We found that anti-CD101 MoAbs inhibited allogeneic mixed dendritic cell-lymphocyte reactions in synergy with anti-CD86 or anti-CD80 MoAbs. Anti-CD101 MoAbs exerted their inhibitory effect at the level of antigen-presenting cells, and not of the responding cells as demonstrated by preincubation experiments. Unlike antiCD86/80 MoAbs, anti-CD101 MoAbs had a direct negative effect on the antigen-presenting cells. Cross-linking of CD101 resulted in a down-modulation of CD86 molecules on skin dendritic cells. Moreover, IL-10 secretion was found upregulated in mixed dendritic-T cells cultures by anti-CD101 MoAbs. Finally a phosphatase activity, that can be partly related to CD148, was found associated to CD101 molecule in monocytes and dendritic cells. Those observations could explain the inhibitory effect of CD101 on T cell activation. Taken together our results suggest a regulatory role played by CD101 molecule on dendritic cells in T cell proliferation.

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Activation of Langerhans Cells Following Transcutaneous Immunization

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We recently reported that cholera toxin (CT) acts as an adjuvant for coadministered antigens following application to intact skin, a method coined transcutaneous immunization (TCI). CT coadministered with bacterial, protozoan, or viral proteins on skin results in potent serum antibody responses to both the adjuvant (CT) and the antigens. While the mechanisms of TCI remain unknown, we hypothesize that Langerhans cells are activated by this method. To address this, the ventral surface of murine ears was treated with CT (250 μg) or an unrelated protein, hen egg lysozyme (250 μg). Flow cytometry of epidermal cell suspensions from CT treated ears showed Langerhans cells with a marked increase in MHC class II and B7-2 and a marked decrease in E-cadherin expression as compared to control ears. Similarly, immunofluorescent staining of epidermal sheets from CT treated ears showed many MHC class II positive cells with morphological characteristics of activated Langerhans cells. Contralateral, vehicle treated ears showed no signs of Langerhans cell activation. Activation of LC in CT treated ears was evident in at 12 h, peaked at 24 h and declined by 48 h. The activation of Langerhans cells appeared to be dose dependent ass lower doses of CT (e.g., 100 μg) induced less Langerhans cell activation as judged by flow cytometry. We conclude that TCI, using CT as adjuvant, activates Langerhans cells in the skin and we hypothesize that Langerhans cells contribute to the ensuing immune response.

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Maturation-Resistant Stably Immature Dendritic Cells Induce T Cell Unresponsiveness *In Vitro* and Prolong Allograft Survival *In Vivo*

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Dendritic cells (DC) were cultured from mouse bone marrow (BM) progenitors in low concentrations of GM-CSF (GM^{lo} DC) by two different protocols. The phenotype and functional properties of these GM^{lo} DC were compared to those of standard BM-DC cultures generated in high concentrations of GM-CSF (GM^{hi} DC) or in low GM-CSF plus IL-4 (GM^{lo}/IL-4 DC). Compared to the latter, GM^{lo} DC were phenotypically immature, weak stimulators of allogeneic mixed leukocyte and oxidative mitogenesis responses, inefficient in peptide presentation to an ovalbumin-specific T cell hybridoma, but substantially more potent in presentation of native protein. Immature GM^{lo} DC were *resistant* to maturation by LPS, TNF-α or anti-CD40 mab, as in contrast to other BM-DC, the expression of costimulatory molecules was not increased, stimulatory activity in oxidative mitogenesis was not enhanced, and secretion of higher levels of IL-12 p40 was not induced. These maturation-resistant, stably immature GM^{lo} DC induced unresponsiveness in allogeneic T cells *in vitro*. GM^{lo} DC also prolonged haplotype-specific cardiac allograft survival (from 8 d to > 100 d median survival time) when they were administered 7 d before transplantation.

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Upregulation of Fcγ Receptors on Epidermal Dendritic Cells is Specific for Psoriasis Vulgaris

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Abnormalities of function and expression of IgG-receptors (FcγR) have been suspected to play a role in the pathogenesis of psoriasis vulgaris (PV). To verify whether this assumption may be relevant for epidermal dendritic cells (DC), we characterized the expression of the high affinity receptor for monomeric IgG (FcγRI/CD64) and the two low affinity receptors for aggregated IgG (FcγRII/CD32, FcγRIII/CD16) on epidermal DC from chronic, untreated PV lesions. Epidermal single cell suspensions were prepared from lesional skin biopsies from n = 41 PV patients. Lesional skin from allergic contact dermatitis (CD; n = 24), atopic dermatitis (AD; n = 63) and normal human skin (NS; n = 38) were used as control. An indirect three color immunostaining for CD1a expression, cell vitality and the respective IgG receptors was done and flow cytometric analysis performed. Fluorescence indices were calculated for quantitative analysis. PV lesions contained significantly less Birbeck granule positive Langerhans cells than NS or AE, and these Langerhans cells expressed significantly more FcγRII than Langerhans cells from AE. In contrast, FcγRI and FcγRIII were not detected on Langerhans cells. In addition to Langerhans cells, a second CD1a positive cell population of the interstitial dendritic cell type was detected in all PV lesions. These cells, previously described as inflammatory dendritic epidermal cells (IDEC), expressed all three IgG receptors. On this IDEC population, both FcγRI and FcγRIII were significantly higher expressed in PV as compared to AD or CD. The expression of FcγRII was also higher, but not significantly. With regard to the respective subpopulations of Langerhans cells and IDEC within all epidermal cells, we established and verified a diagnostic algorithm on the basis of FcγRIII expression. In conclusion, high Fcγ receptor expression seems to be highly specific for PV. This observation indicates that (i) the analysis of Fcγ receptor expression on epidermal DC could be useful for diagnostic purposes in PV and (ii) these structures may play a pathophysiological role in this disease.

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Regulation of IL-10 Secretion and mRNA Expression by 1,25-Dihydroxyvitamin D_3 and its Analogue Tacalcitol in Monocyte-Derived Dendritic Cells

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Psoriasis is believed to be a Th1-mediated disease in which cytokine dysbalance is characteristic. Studies indicate that the activation of lesional T-cells may be caused by (auto)antigens and mediated through antigen presenting cells. Dendritic cells are antigen presenting cells playing an important role in the immuno pathogenesis of psoriasis. The use of vitamin D-analogues in the treatment of psoriasis has been an important new development, but the mechanisms of action of these drugs are not fully understood. Therefore, the possible modulatory role of 1,25-Dihydroxyvitamin D_3 (calcitriol) and its analogue tacalcitol on IL-10 secretion and expression by monocyte-derived dendritic cells was investigated in our study.

Monocyte-derived dendritic cells (MoDC) were obtained by incubation of purified human monocytes in RPMI-1640 supplemented with GM-CSF (100 U per ml) and IL-4 (10 ng per ml) for 5 d. Calcitriol, tacalcitol and $24,25(OH)_2D_3$ were dissolved in ethanol and added to the MoDC cultures at a concentration of 10^{-8} M from the beginning. Calcitriol was used at concentrations from 10^{-8} to 10^{-13} M. After 5 d of culture MoDC were harvested and mRNA expression for IL-10 was analysed by semiquantitative RT-PCR. IL-10 protein secretion was measured in the culture supernatants using specific ELISA.

The results of the study show that calcitriol and tacalcitol enhanced the production of IL-10 at the levels of both, mRNA expression and protein secretion in a dose-dependent manner. The solvent ethanol as well as $24,25(OH)_2D_3$, an analogue of calcitriol with low affinity to vitamin D receptor, showed no effect on IL-10 production.

It has been previously shown that psoriatic skin-derived dendritic cell function (T cell stimulating ability) is inhibited by exogenous IL-10. Furthermore, IL-10 treatment of psoriasis patients showed improvement of lesions. Therefore, the findings of our study indicate that the therapeutic effect of calcitriol and its analogues in psoriasis may at least in part be mediated through induction of IL-10 production.

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CD45RA-Expressing Malignant T-Helper Cells in Sezary-Syndrome (SS)

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Sezary syndrome (SS), a primary cutaneous T-cell lymphoma, is characterised by erythroderma and generalised lymphadenopathy, furthermore by the existence of neoplastic T-cells in skin, lymph nodes and blood (>1000 Sezary cells per mm^3). Until now, the malignant T-cell was believed to be of "memory" phenotype, characterised by the expression of CD3+, CD4+, CD45RO+. Our own investigations, including four patients with SS, showed a more heterogenous phenotype of the malignant cell, which in two cases turned out to be predominantly CD45RA+ with a high percentage of them being CD45RA+ CD45RO+ double positive. The malignant cells of only two of the patients showed the characteristics of a "memory phenotype", expressing CD3+, CD4+, CD45RO+. Moreover, the phenotype of the malignant cells, designated by the expression of a certain TCR-Vβ, was characterised by a high percentage of CD62 L-(L-Selectin, LECAM-1) expressing cells, lacking activation-antigens like CD25 or HLA-DR. All of them expressed CD28, whereas only a small percentage of neoplastic cells showed expression of CD26. Moreover the expression-level of CD95 was dramatically decreased, which implies a reduced susceptibility to apoptosis. Our studies were based on flow cytometric characterisation, and for the first time, it enabled us to demonstrate the malignant T cell in CTCL to differ from "memory" phenotype. These findings are particularly important, since the memory phenotype of the malignant cell was suggested to be a consequence of activation during transformation, necessary for their homing to the skin. According to our data CD45RO-expression of malignant T-cells in CTCL might not be an essential characteristic of skin homing neoplastic T-lymphocytes in CTCL.

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Telomerase Activity is Increased and Telomere Length Shortened in T Cells from Patients with Atopic Dermatitis and Psoriasis

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We studied telomerase activity and telomere length in peripheral blood mononuclear cells (PBMC), purified $CD4^+$ and $CD8^+$ T cells from blood, and in skin-homing T cells from a total of 37 patients with atopic dermatitis, 12 patients with psoriasis as well as 22 age matched normal donors. We used the telomeric repeat amplification protocol based telomerase PCR ELISA for assaying telomerase activity, and Southern blot analysis for measuring telomere length. The telomerase activity was significantly increased in PBMC from the patients compared to PBMC from normal donors. This increase was most pronounced in the $CD4^+$ T cell subpopulation. Telomerase activity was also elevated in skin-homing T cells. The telomere length was significantly reduced in all cell types from both atopic dermatitis and psoriasis patients compared with normal individuals, but did not differ between diseases. Skin-homing T cells did also have shortened telomeres compared with blood T cells obtained from healthy persons. In conclusion, the increased telomerase activity and shortened telomere length indicates that a significant subgroup of circulating T cells in atopic dermatitis and psoriasis are at a different developmental stage and/or with an increased cellular turn-over *in vivo*. It is likely that skin-homing T cells in both diseases are derived from this subpopulation.

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Allergen Uptake by Dendritic Cells Generated from Cord Blood Progenitors

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The safety and efficacy of sublingual immunotherapy have been demonstrated in moderate allergic asthma and seasonal rhinitis. In order to define the precise action mechanism of the allergen when it crosses the oral mucosa, we investigated the role of Langerhans cells in the capture and internalisation of allergens.

We generated *in vitro* dendritic cells with the phenotypic characters of Langerhans cells (LLDC) from cord blood CD34+ progenitors cultured with GM-CSF and TNFα. We used two recombinant major allergens: Birch pollen allergen 1 (r-Bet v 1) and Phleum pratense allergen 1 (r-Phl p 1) labelled with FITC. As control, we used a panel of FITC labelled proteins matched for molecular mass or the absence of glycosylation. Dextran-FITC was used as control for endocytosis via receptors.

Internalisation of allergens as other proteins was dose and time dependent and was not saturable. Allergens were mainly internalised by LLDC whereas Dextran-FITC was internalised by LLDC as well as by non Langerhans like DC.

LLDC use distinct mechanisms for antigen capture. Allergens were only internalised by macropinocytosis as demonstrated by the use of various inhibitors. LLDC which are immature cells internalised allergens by macropinocytosis with a high efficiency indicating by this way that macropinocytosis is constitutive in contrast to other cells such as monocytes. By confocal microscopy, we showed that allergens were internalised by the cell body and the dendritic processes. Addition of monensine indicated that part of allergens was accumulated in lysosome whereas the major part stayed in cytoplasmic structures. Pulse-chase experiments allowed to calculate a half life of 3 h. These data suggested that part of the molecules were not metabolised in the lysosome but must be directly released in the medium.

Allergen internalisation by LLDC might be followed by a processing as demonstrated by activation of autologous T lymphocytes in three experiments over a total of seven.

These elements showed that Langerhans cells in mucosa may play an active role in primary immune responses to allergens.

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Fragrance and Contact Allergens *In Vitro* Modulate the HLA-DR and E-Cadherin Expression on Human Epidermal Langerhans Cells

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Epidermal Langerhans cells play a critical role in the induction of contact hypersensitivity (HSR). The Langerhans cells leave the skin, move to the regional lymph nodes and present the allergens embedded in the HLA-DR molecule to naïve T-lymphocytes. To allow for Langerhans cell emigration from the epidermis, E-cadherin must be down-regulated. In this study, we have examined alterations of E-cadherin and HLA-DR expression after exposure to three strong contact sensitizers and two commonly used fragrances. Flow cytometry was utilized to evaluate E-cadherin and HLA-DR expression on human epidermal Langerhans cells exposed to the different chemicals for 4 h at 37°C. *In vitro* stimulation with the contact sensitizers isoeugenol, cinnamaldehyde, 2,4,6-trinitrobenzenesulfonic acid (TNBS), Bandrowski's base (BB), or *p*-phenylene diamine (pPDA) resulted in a dose-dependent decrease of HLA-DR expression on the surface of Langerhans cells without affecting the number of positive cells. These contact allergens induced a down-regulation of E-cadherin expression as well as a significant decrease of the percentage of E-cadherin positive cells. Incubation with an irritant, sodium lauryl sulfate (SLS), did not significantly change HLA-DR and E-cadherin expression. Based on the alteration of E-cadherin and HLA-DR expression of human Langerhans cells under short-term exposure conditions, there was a clear difference between contact sensitizers and a well characterized irritant. For the first time, the ability of fragrance allergens in dipropylene glycol (DPG), a widely used vehicle in fragrance and cosmetic industries, was demonstrated to induce human Langerhans cell phenotypic alterations. In combination with a series of *in vitro* tests, this rapid and simple method should help to detect the sensitizing potential of a substance to be applied onto the human skin as an alternative to animal testing.

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Normal Human Keratinocytes (KCs) Express CD1d

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CD1d-restricted T cells are suspected to play important roles in the control of autoimmune disease and the immune response to tumors. The tissue distribution and cell type specific expression of human CD1d remains incompletely characterized, although initial reports have emphasized its expression by intestinal and thymic epithelia. In the present study, we investigated whether CD1d was also expressed on skin epithelial cell-derived KCs. CD1d expression by multipassaged human KCs and HaCaT cells (a transformed KC cell line) was assessed at the mRNA and protein level. Using highly specific primer sets, CD1d mRNA was constitutively detected in both KCs and HaCaT cells, but not in cultured fibroblasts using RT-PCR or other control cell lines. CD1d protein was also demonstrated on Western blot analysis in HaCaT and KCs, but was absent from control cell lines that lacked CD1d-specific mRNA. The size of protein expressed by KCs (approximately 45 kDa) was distinct from the previously reported size of CD1d molecules expressed on intestinal epithelial cells (37 kDa) suggesting that in KCs CD1d is glycosylated. Immunostaining of human skin and Kcs grown as monolayers on Lab Tek Chamber slides using a panel of 20 different mAbs specific for CD1d revealed various patterns of staining (i.e., plasma membrane and/or cytoplasmic staining, distributed either to the full thickness of the epidermis or sparing the basal layer). This diversity may reflect various post-translational and potentially functionally distinct states of the molecule in the various layer of the epidermis. Cell surface expression was confirmed by flow cytometry, and pretreatment of KCs and HaCaT cells with IFN-γ or high calcium concentrations (2 mM) increased CD1d expression compared to isotype control staining. In conclusion, we have shown that CD1d can be expressed by epidermal KCs. CD1d expressed by these cells appeared to be normally glycosylated, and its cell surface expression was upregulated by IFN-γ and calcium. We hypothesize that cutaneous immunohomeostasis may in part be regulated by a subset of CD1d-restricted T cells interacting with benign and/or malignant KCs.

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The Maturation Level of Dendritic Cells (DC) Modifies their Sensitivity to Melanoma-Induced Apoptosis

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We have recently demonstrated that both murine and human tumors induce premature apoptosis of DC. Since intratumoral accumulation of DC is associated with prolonged survival and a reduced incidence of metastatic disease in melanoma, prevention of tumor-induced DC apoptosis might represent a novel treatment strategy. We have tested the hypothesis that mature DC are more resistant to melanoma-induced apoptosis compared to immature DC. Murine DC were cocultured with CD154(CD40L)-transfected or nontransfected fibroblasts for 24 h prior to labeling with thymidine. ^{3}H-DNA-releasing assays using B16 melanoma as effector cells revealed that CD40 ligation protected DC from melanoma-induced apoptosis. Activation of DC using TNF-α also resulted in enhanced resistance of murine DC to B16 melanoma induced apoptosis *in vitro*. This effect was accompanied by an up-regulation of the apoptosis inhibitory protein Bcl-2 and a down-regulation of the apoptosis promoting protein Bax in DC as was assessed by Western blot. These data suggest that activated DC are less sensitive to melanoma-induced apoptosis. Furthermore, Bcl-2 is important for DC survival and might represent a target for strategies designed to protect DC from tumor-induced apoptosis. Thus, mature DC do have a survival advantage within the melanoma microenvironment. This finding suggests that mature DC might be more effective in clinical trials.

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Interactions of Murine Dendritic Cells with *Leishmania major*

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L. major amastigote-infected C57BL/6 (B6) fetal skin-derived DC (FSDDC) are activated, release cytokines (including IL-12 p70) and likely initiate protective Th1 immunity in resistant B6 mice (*J Exp Med* 188:1547). To determine if there are differences in DC function in mice that are genetically susceptible (BALB/C) and resistant (B6) to leishmaniasis, we analyzed the effects of *L. major* on FSDDC from both strains. Infection of BALB/C and B6 DC led to upregulation of MHC class 1 and 11 Ag and co-stimulatory molecules (CD40, CD54, CD80 and CD86) within 18 h. *L. major*-induced BALB/C (and B6) DC activation caused release of similar amounts of TNFα, IL-6 and IL-12 p40 into 18 h supernatants. Infected BALB/C and B6 DC also released IL-12 (p70) into 72 h supernatants (35 ± 28 *vs* 4 ± 2 pg per 10^6 DC, n = 6). Additional stimulation with IFHγ, anti-CD40 or both induced the release of more p70 from infected BALB/C DC (123 ± 78 116 ± 53 and 244 ± 103 pg per 10^6 DC, n = 6) than B6-DC ($25 \pm 7{,}23 \pm 8$ and 52 ± 8 pg per 10^6 DC, n = 8). Co-culture of infected BALB/C and B6 DC with naïve syngeneic $CD4^+$ T cells and soluble anti-CD3 resulted in a mixed, IFNγ-predominant response after restimulation with immobilized anti-CD3. Thus, activation of skin DC from genetically susceptible and resistant mice by *L. major* induced release of similar amounts of IL-12 and led to a mixed, Th1 predominant immune response in BALB/C and B6 T cells *in vitro*. Preferential induction of Th2 predominant responses to *L. major* in BALB/C mice does not appear to reflect the inability of DC/Langerhans cells to internalize or respond to parasites or to initiate Th1 responses in naïve $CD4^+$ T cells *in vitro*. Susceptibility in BALB/C mice could reflect a defect in recruitment of DC in the initiation phase of the immune response or result from an abnormality not manifested in DC.

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HLA-DM and HLA–DO are Expressed on Keratinocytes in Psoriasis Vulgaris but not Normal Human Skin

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In B-cells, the nonclassical human leukocyte antigens HLA-DM (DM)and HLA-DO (DO) are residents of lysosome-like organelles where they form tight complexes. DO preferentially promotes loading of HLA class-II molecules that are dependent on the chaperone activity of DM. To explore a potential role for these molecules in cutaneous inflammation we analyzed their expression in human epidermis. Immunohistochemical staining of biopsy material from normal human skin revealed only a weak expression of DM in the granular layer of the epidermis. In contrast, lesional psoriatic skin showed a strong expression of DM as well as a neo-expression of DO. The former is expected since keratinocytes in lesional psoriatic skin express HLA class-II molecules, whereas expression of DO in nonprofessional antigen presenting cells has not yet been reported. Western blot analyses of psoriatic epidermis confirmed the presence of DO. DO expression was not detected in positive patch tests thus excluding that this is a general feature of cutaneous inflammation. This is the first report on the expression of DO on nonprofessional antigen presenting cells. Since DO is involved in peptide editing ist expression in psoriatic lesions might be relevant for the generation of a T-cell mediated autoimmune response.

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Functional Characterization of the Novel CD8+ T Lymphocyte Subset Lacking Both CD28 and CD11b Markers

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T lymphocytes are crucially involved in cutaneous biology, since they show a special affinity for the skin. We recently identified, both by panning methods and by triple-color FACS analysis, a novel subset of CD8+ T lymphocytes lacking both the CD28 and CD11b markers, namely the CD8+ CD28–CD11b-subpopulation. In the present study we intended to investigate the functional properties of such a novel T cell subset. Since both the CD8+ CD28– (CD11b+) and the CD8+ CD11b– (CD28+) subsets exert cytolytic capability, we first tested, using P815 targets in a [^{51}Cr] release assay, the cytolytic ability of the CD8+ CD28– CD11b– cells, sorted from peripheral blood obtained from 50 healthy volunteers. Surprisingly, such cells lacked any cytolytic effect; they were moreover not able to proliferate after stimulation with PHA plus IL-2, as detected at 90–94 h by [^{3}H] thymidine uptake. Cells resulted, however, not apoptotic, as defined by TUNEL reaction; their biological activity was moreover confirmed with the aid of RT-PCR amplification analysis, because they, even if unstimulated, expressed high levels of IFN-γ and TNF-α mRNA (but not of IL-2 and IL-4 mRNA). In conclusion, these data suggest that the CD8+ CD28– CD11b– subset is somehow aberrant, in that it lacks both proliferative and cytolytic capacities which are prerequisites of the virtually total CD8+ population; although it might therefore be considered a terminally differentiated subpopulation, it should not represent, however, an end-stage subset, because is not apoptotic and expresses high levels of proinflammatory cytokines mRNA.

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Evolution of T-cell Repertoire in Treated Sezary Syndrome

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Sezary syndrome (SS) is a leukemic form of cutaneous T-cell lymphoma related to the malignant proliferation of clonal CD4+ T-cells. Extracorporeal photopheresis (ECP), interferon alpha (IFNα) or antineoplastic polychemotherapy may induce clinical improvement in some cases. Follow up quantitative molecular analysis of the malignant clone has never been reported. In order to investigate the representation of the T-cell clone in the peripheral blood of 11 patients with SS, receiving treatment with ECP (eight patients), IFNα (one patient) and CHOP (two patients), we used a semiquantitative technique based on RT-PCR BV-BC and immunoscope determination of the CDR3 length. As previously reported, we did not find any BV preferential expansion. We showed that the percentage of Sezary cells was not correlated to the relative frequency of the clone. One of the eight patients treated with ECP showed a decrease of the relative frequency of the T-cell clone from 15,6% to 0%, which was correlated to the complete clinical remission of the disease. In the seven remaining cases showing no clinical improvement, weak changes in the relative frequency of the dominant BV-BC rearrangement were observed. In one patient showing a partial remission following polychemotherapy, a decrease of the relative frequency of the clone from 43% to 7% was observed. In the other chemotherapy-treated case, the relative frequency of the clone was stable in parallel to the absence of any clinical benefit of the treatment. Finally, the clinical status of the IFNα-treated patient improved whereas an increase of the relative frequency of the clone from 15% to 51% was observed, in parallel to a disappearance of the non tumoral polyclonal lymphocytes.

In conclusion, RT-PCR BV-BC and immunoscope analysis of the CDR3 represent a semiquantitative technique that may be applied to the follow-up of the T-cell clonal component in SS. In our study, the evolution of the relative frequency of the clone seemed to parallel the clinical course of the disease in patients treated with ECP and polychemotherapy.

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Major Histocompatibility Complex Class I-Restricted CD8+ Cells are Involved in UVB-Induced Immunosuppression of Contact Hypersensitivity Response to Dinitrofluorobenzene

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Cutaneous exposure to ultraviolet radiation (UVR) impairs the induction of contact hypersensitivity (CHS) response to haptens applied directly to the irradiated skin surface in certain strains of mice and in humans. Both CD4+ and CD8+ T-cells can transfer suppression depending on the experimental model used. To investigate more precisely the relative contribution of these two cell subtypes, we used C57BL/6 mice deficient in invariant chain (Inv°) and thus deficient in CD4+ T-cells, in a model of CHS to dinitrofluorobenzene (DNFB) in which CD8+ T-cells are effector cells of CHS. In addition, knockout C57BL/6 mice with a mutation in the Aβ gene or in the perforine gene and CH3/HeN-*gld* mice were used. Mice were exposed to 800 or 1200 J per m^2 of UV on the shaved back daily for four consecutive days. They were then sensitized with DNFB on the irradiated skin, and challenged on both UV-protected ears. Our results showed that Inv° mice as well as perforine and FasL-deficient mice demonstrated an inhibition of CHS response to DNFB following UVB irradiation. Our data indicate a crucial role for CD8+ T cells in UVB-induced immune suppression of CHS response to DNFB, whereas the apoptosis-associated perforine and Fas ligand systems were not involved.

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α1,3-Fucosyltransferase VII mRNA Expression is Induced by Superantigen Stimulation and Inhibited by N-Acetylcysteine

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Cutaneous lymphocyte-associated antigen (CLA), the most relevant adhesion molecule for the recruitment of T-cells into the skin, has recently been suggested to be produced by post-translational glycosylation of P-selectin glycoprotein 1 (PSGL-1) by the action of the a1,3-fucosyltransferase VII (FucTVII). a1,3-Fucosyltransferases are considered to be the key enzymes, which catalyze the transfer of fucose to *N*-acetylglucosamine via an a1,3-linkage. We and others have shown that superantigens are strong inducers of CLA on T-cells. We therefore asked if superantigens would also induce FucTVII expression. FucTVII is only weakly expressed in resting PBMCs. Activation with the superantigen TSST-1 (100 ng per ml) induced a marked upregulation of FucTVII mRNA as determined by RT-PCR and identification by sequence analysis. The maximum FucTVII mRNA levels were reached at 48 h after stimulation, approximately another 48 h before maximum expression of CLA and maximum binding to E-selectin as determined by *in vitro* binding and intravital mouse-ear microscopy. Furthermore, we had previously shown that N-acetyl-L-cysteine (NAC) reduces the expression and function of CLA. This downregulation by NAC seems based on its antioxidant properties since the unrelated antioxidants α-tocopherol and α-lipoic acid also significantly reduced CLA expression. The FucTVII mRNA expression of TSST-1 activated PBMCs could be inhibited by a combination of NAC (25 mM), α-tocopherol (50 μM), and α-lipoic acid (100 μM). Since FucTVII has several putative, redox-sensitive transcription factor binding sites in its promoter region, it is tempting to speculate that these could be the site of action for the inhibition of FucTVII and as a result of CLA. Indeed, TSST-1 activates the redox sensitive transcription factor NF-κB in PBMCs. Our results suggest that both the expression of CLA by superantigens as well as the inhibition of CLA by antioxidants is regulated at the transcriptional level of FucTVII via NF-κB activation. This understanding may provide the basis for novel therapeutic approaches in the treatment of T-cell mediated dermatoses.

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In Vitro Induction of Melanoma-Specific CTL Clones From PBL

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In order to derive Melan-A/MART-1 specific T cell populations from the blood of melanoma patients, we extensively compared the efficiency of various *in vitro* stimulation protocols by Melan-A/MART-1 peptides.

We first show that peptide-pulsed allogeneic melanoma cells are more efficient than peptide-pulsed autologous PBL in stimulating highly reactive Melan-A/MART-1 specific T cells. Such specific T cells could be obtained systematically from all HLA-A*0201 donors (healthy or melanoma patients). We also show that a modified analogous peptide enhanced the growth of specific T cells and allowed to reduce cytokine used during stimulation protocols. In addition, once isolated by tetramer labeling or by cloning, specific T cells exhibited a high reactivity against tumor cells, as indicated by their level of lysis and of cytokine secretion, including IL-2. Since T cell clones generated by this method had a high growth capacity, we could settle a process that allows to produce high numbers of specific T lymphocytes to be used for immunotherapy from most HLA-A*0201 donors.

Furthermore, since Melan-A/MART-1 is expressed by most melanoma tumors, these clones could be produced and used for adoptive immunotherapy in most HLA-A2 patients.

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TCRBV Repertoire Analysis of T Cells Infiltrating Skin Lesions in HIV-Related Psoriasis: Evidence for a Compartmentalized, Antigen-Driven T Cell Expansion

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One of the main features of psoriasis occurring in HIV-infected patients is the predominance of CD8+ cells among T lymphocytes infiltrating both dermis and epidermis, while CD4+ T cells are predominant in the dermal infiltrate in psoriasis occurring in HIV seronegative patients. In order to investigate the mechanisms involved *in vivo* in the skin-localized expansion of CD8+ T lymphocytes, we have used a semiquantitative RT-PCR based method for a comparative analysis of the TCRBV CDR3 length distribution in both the skin infiltrate and the purified CD8+ blood lymphocytes of HIV-infected patients affected with psoriasis.

In all patients included in this study, immunohistochemical analysis of frozen skin biopsies revealed that activated CD8+ T cells expressing a cytotoxic phenotype were strikingly predominant among the dermal/epidermal infiltrate, as shown by reactivity with anti-TiA-1 and antiperforin monoclonal antibodies. RT-PCR based analysis of the repertoire of skin-infiltrating cells showed oligoclonal patterns in most TCRBV families analyzed, with dominant peaks showing a unique CDR3 length in some cases. When performed, cloning and further sequencing of dominant peaks demonstrated the monoclonality of these skin CD8+ expansions. Furthermore, while peaks of identical CDR3 lengths were found in both CD8+ peripheral blood lymphocytes and in the skin, other peaks were found specifically expanded in the skin lesions. Finally, the TCRBV CDR3 length distribution was investigated in two biopsies concomitantly taken from two distinct plaques.

Altogether, these data suggest that the activation and the expansion of cutaneous CD8+ cytotoxic T lymphocytes in the lesions of HIV-associated psoriasis may be driven by TCR-dependant, antigenic stimuli possibly exhibiting spatial discontinuities.

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Human T-Lymphocytes Express the C3a Receptor

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The C3a molecule is an anaphylatoxin of the complement system with various potent functional effects mainly on cells of myeloid origin. In this study we investigated the expression of the C3aR on human T-lymphocytes both on the mRNA and on the protein level. We found no or only low expression of C3aR mRNA and no or only low binding of monoclonal anti-C3aR antibodies in unstimulated freshly isolated T-lymphocytes from healthy donors. In contrast, circulating T-cells from patients suffering from disseminated severe inflammatory skin diseases (i.e., pemphigus foliaceus, psoriasis erythrodermia, acute eczema, erysipelas) showed an expression of C3aR on T-cells. An incubation with type I interferons (but not with type II interferons, IL-4, IL-5, IL-6, IL-8, IL-10 or IL-12) led to the induction of C3aR on T-cells *in vitro*. Interestingly, we found C3aR+ skin-infiltrating T-lymphocytes at the sites of IFN-β injections during the treatment of multiple sclerosis. A high expression of C3aR was detected on a number of CD4+ or CD8+ T-cell clones (TCC) from patients with atopic dermatitis or allergic contact dermatitis both on the mRNA and on the protein level. The binding of anti-C3aR antibodies to these TCC was specific since it could be blocked with a peptide representing the recognized C3aR sequence. Moreover, C3a led to a downmodulation of the C3aR upon incubation over 20 h. C3a led to a transient calcium influx in C3aR+ TCC which could be blocked by preincubation of the stimulus with anti-C3a antibodies. First functional experiments showed an upregulation of IL-10 in C3a-treated T-lymphocytes. Taken together, we provide direct evidence for the expression of C3aR on human T-lymphocytes for the first time which may point to a biological function of C3a in T-cell regulated dermatological diseases.

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In Vivo Immunological Effects of Photopheresis in a Mouse Model

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The treatment of cutaneous T cell lymphoma with photopheresis combines the administration of 8-Methoxypsoralen (8-MOP) with subsequent leukapheresis, UVA irradiation and reinfusion of the cells. In the course of such a treatment photochemically altered tumor cells seems to induce an antitumoral effect. In order to elucidate the underlying immunological mechanism of this therapy we established a mouse lymphoma model. This enabled us to compare immunization with photochemically treated cells to immunization with gamma-irradiated cells and to lysed tumor cells. Both photochemically altered and gamma-irradiated syngeneic tumor cells generated an immune response protective against subsequent lymphoma challenge, whereas immunization with tumor lysates did not. We show that the photochemical treatment with 8-MOP plus UVA and gamma-irradiation have in common the ability to induce of the lymphoma cells, which appears to be a prerequisite for successful immunization, since apoptotic cells are known to be efficiently processed by professional antigen presenting cells.

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T-Cell Response Patterns in T-Cell Reactive Leprous and Sarcoid Granulomas

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A variety of mycobacterial antigens including peptides, lipids and glycolipids is responsable for the divers nature of the cutaneous immune response in the T-cell reactive forms of leprosy with stimulation of α/β T-cells, γ/δ T-cells, and NKT-cells. The latter NKT-cells which carry a conserved TCRVα-Jα rearrangement with only limited Vβ variation have been shown to have a pivotal role in the development of granulomas induced by lipophilic mycobacterial cell wall extracts in an animal model.

In order to study the role of NKT cells in the formation of cutaneous granuloma of infectious and idiopathic origin and to study the nature of antigenic stimulation of α/β T-cells in these conditions we took biopsies from six patients with cutaneous sarcoidosis and compared them with five biopsies of patients with tuberculoid leprosy, three patients with lepromatous leprosy, two patients with the reversal form and one normal skin biopsy by analysing the invaded T cell populations using immunohistochemistry, RT-PCR, the Immunoscope technique and sequence analysis. This approach showed restricted Vα usage in sarcoidosis as well as in leprosy granulomas. In sarcoidosis an antigen-driven-like pattern could be found with different V α bearing T cells on expansion. Sequence analysis of the CDR3 region of these peaks showed an individual dominant sequence in the majority of patients which was not shared between patients. In tuberculoid and reversal leprosy patients besides the expected finding of Vα 24 positive cells a strong bias towards Vα 6 and Vα 14 with a polyclonal expansion pattern could be detected. CDR3 sequence of these expansions revealed oligoclonal expansions with repetitive sequences which were not shared between the patients. We could identify in all leprosy patients with active cellular immune response the canonical Vα24-Jα18 rearrangement of the α/β TCR which is typical for human NKT-cells. Moreover six of seven patients scored positive for Vα 24 positive T-cells in the immunohistochemistry. Sarcoidosis patients, however, were found negative for the presence of NKT cells by both techniques. In summary we could identify NKT-cells in the investigated infectious leprous granuloma lesions but not in the sarcoidosis patients. Analysis of the TCR α chain in sarcoidosis suggests an antigen driven process with individual clones on expansion. In contrast the investigated leprosy patients showed biased TCR Vα chain usage with oligoclonal expansions mainly for Vα 6 and Vα 14.

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Glucocorticoids Differentially Inhibit the TGF-α- and HGF/SF-Induced VEGF/VPF Expression by Cultured Keratinocytes

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Regulation and expression of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) has been demonstrated to be critical for both physiologic and pathophysiologic angiogenesis. Besides tissue hypoxia, in particular the cytokines transforming growth factor (TGF)-α and the hepatocyte growth factor/scatter factor (HGF/SF) potently induce VEGF/VPF gene expression by primary keratinocytes. Since angioproliferation and vascular hyperpermeability represent prominent components of cutaneous inflammation, we proposed that the known anti-inflammatory properties of glucocorticoids are attributed in part to their interference with the regulated VEGF/VPF expression by keratinocytes. We thus examined the effects of glucocorticoids as known potent inhibitors of inflammatory processes on the TGF-α- and HGF/SF-induced VEGF/VPF expression by keratinocytes *in vitro*. Our studies reveal significant inhibitory effects of different structurally unrelated glucocorticoids on the induced VEGF/VPF expression by keratinocytes *in vitro*. Both the TGF-α- and HGF/SF-induced VEGF/VPF protein and mRNA expression are inhibited in a concentration-and time dependent fashion. The two molecular pathways studied exhibit a distinct sensitivity to glucocorticoids. Whereas HGF/SF-mediated VEGF/VPF induction is entirely blocked, TGF-α-induced VEGF/VPF expression is suppressed by >>30%, demonstrating differential effects of glucocorticoids on TGF-α- and HGF/SF-mediated VEGF/VPF synthesis. In the absence of a significant increase in VEGF/VPF mRNA stability as a major determinant of mRNA abundance and subsequent gene expression, both TGF-α- and HGF/SF-mediated VEGF/VPF mRNA up-regulation appear to be primarily dependent on enhanced transcriptional activation. Thus, interference with the induced VEGF/VPF expression by glucocorticoids is most likely conveyed by molecular mechanisms involving gene's transcriptional activation. Together, our studies highlight angiogenesis as an additional target for glucocorticoid action in cutaneous inflammation.

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Polyclonal Expansion of Vβ Restricted T Cells Indicates Involvement of Superantigen in the Pathogenesis of Cutaneous T Cell Lymphoma

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Mycosis fungoides (MF) is a tumour of low grade malignancy which is characterised by a strong epidermal and dermal lymphocytic infiltrate. Staining for the TCR-Vβ chains often shows an expanded Vβ in the infiltrate. These cells are believed to be the tumour cells. In the present study the infiltrating T cells isolated from skin biopsies of one patient were investigated for their Vβ phenotype and genetically for their clonotypic TCR-γ rearrangement. The skin infiltrating T cells were cloned and analysed by flow cytometry for the expression of Vβ5.1 which is over-represented in the epidermis of the patient. 201 cell lines and clones could be established from a punch biopsy and 493 of a aspiration biopsy, of which 21% and 67% were Vβ5.1 positive, respectively. 204 of these $V\beta5.1^+$ clones were analysed for the sequence of the junctional region or their TCR-γ after PCR amplification. When compared to the sequence of the expanded T cell clone found in the primary diagnostic clonality analysis, none of the cultured cells were the tumour cell clone. All but two of these clones were different indicating that the $V\beta5.1^+$ cells in the epidermis of the MF patient are polyclonal. In a second line of investigations the clonotypic TCR-γ rearrangements of microdissected single cells picked from an independent tissue specimen of the same patient were analysed. These analyses revealed that about 70% of the $V\beta5.1^+$ cells in the epidermis belong to the tumour clone.

It can be concluded that in cases of MF the skin infiltrating T cells which express the same TCR-Vβ are polyclonal with the putative tumour clone belonging to this population. These results suggest involvement of superantigen in the pathogenesis of the disease. It needs to be investigated whether superantigen plays a role in its initiation and its aetio-pathogenesis or act at later stages.

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Overexpression of hsp25 in K1735 Murine Melanoma Cells Stimulates Natural Killer Cytotoxicity

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It has been previously shown in human melanoma and squamous cell carcinoma cell lines that overexpression of the small heat shock protein hsp27 inhibits cell growth and tumorigenicity. In the present study we used a murine melanoma model to investigate the effect of hsp25, the murine homologue of hsp27, on natural killer cytotoxicity. The murine melanoma cell lines K1735-Cl23 (low metastatic potential) and K1735-M2 (high metastatic potential; both lines were a kind gift of I.J.Fidler, Houston, TX) were transfected with *hsp25* under the control of a retroviral LTR promotor. Stable transfectants expressing high levels of hsp25 were cloned and analyzed by immunoblotting. Control clones were obtained by transfection with antisense-*hsp25*. Spleen cells of syngeneic C3H/HeN mice were used as effector cells in a 4-h cytotoxicity assay to test the susceptibility of K1735 sublines to natural and IL-2 activated lymphocyte cytotoxicity. Hsp25 overexpression was associated with increased susceptibility to spontaneous and IL-2 enhanced cytotoxicity in K1735-Cl23. In contrast, K1735-M2 was resistant to natural cytotoxicity and hsp25 overexpression could not overcome this resistance. In summary, tumor cell expression of small heat shock proteins can enhance natural killer cytotoxicity in murine melanoma. Further investigation of the molecular mechanisms of this effect in the described model of "hsp25-susceptible" and "hsp25-resistant" melanoma phenotypes might help to develop novel means to enhance immunological tumor surveillance.

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High Frequency of Dominant T Cell Clone Detection by Heteroduplex Analysis in Cutaneous Lesions of Pityriasis Lichenoides et Varloliformis Acuta (PLEVA)

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Cutaneous lesions of PLEVA, a T cell mediated cutaneous inflammatory condition, are clinically very similar to lymphomatoid papulosis (LyP), leading some authors to hypothesize they are part of the same pathological spectrum of lymphoproliferative disorders. However, unlike LyP, no systematic search for a dominant T cell clone has been carried out in PLEVA whereas clones have been detected in scattered cases using Southern blot methods.

Twenty clinically and histologically typical cases of PLEVA were selected for this study. Genomic DNA was extracted from archival paraffin-embedded biopsies after wax removal, amplified by PCR with primers for the V regions Tγ1–8, Tγ10, Tγ10/11, Tγ11 and for the Jγ1 region, and finally submitted to heteroduplex analysis, PBMC of a healthy control and Jurket cells were used as negative and positive controls, respectively. Successive dilutions of DNA from Jurkat cells in DNA of PBMC evaluated the sensitivity of this method of detection of a dominant T cell clone. Thirteen out of 20 cases (65%) of PLEVA cases displayed the presence of a dominant T cell clone by this method. Positive and negative controls confirmed its specificity. Its sensitivity was determined to be between 1 and 5% of the total T cell infiltrate.

This study gives further support to the hypothesis that PLEVA may be, in some if not in the majority of cases, included in the spectrum of clonal T cell lymphoproliferative disorders.

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Failure of Adjuvant High Dose IFNα2b Therapy of Melanoma Associated with Altered Expression of Classical and Aberrant Expression of Nonclassical HLA Molecules Prior to Therapy

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By demonstrating favorable effects on relapse free and overall survival of lymph node-positive melanoma patients postsurgery high dose IFNα therapy gave rise to hope for an effective adjuvant therapeutic strategy in malignant melanoma. Recently, this enthusiasm was followed by disillusion due to an interim analysis of the successor E1690 trial that failed to confirm impact on overall survival. Nevertheless, there are patients who clearly benefit from this therapy and others who don't, but individual predictive clinical, immunological, or molecular features for definition of those patients are lacking.

The most important biological effects of IFNα constitute upregulation of HLA molecules on tumor cells, induction of a Th1-biased immune response, and activation of natural killer cells. By loss of classical HLA molecules melanoma cells can escape CTL immunosurveillance but become natural killer celltargets. However, additional expression of the nonclassical class 1 molecule HLA-G on melanomas can lead to escape from natural killer cell immunosurveillance by interaction with natural killer-inhibitory receptors.

To analyze the expression of classical and nonclassical HLA-molecules on melanoma cells metastatic to the locoregional lymph node prior to therapy for correlation with failure to subsequent high-dose IFNα2b therapy, we used an immunohistochemical technique adapted to retrieve cell surface antigens on archival tissue specimens. Results obtained with this technique correlated well with control biochemical immunoprecipitation/SDS-PAGE analysis of HLA-expression. In contrast to patients without relapse we observed expression of HLA-G on melanoma cells in five of five specimens with total loss of HLA-A, -B, -C molecules in two of five and focal loss in three of five samples of patients facing a relapse under therapy.

Whereas some HLA phenotypes with locus-specific transcriptional downregulation may be overridden by IFN treatment, HLA phenotypes due to structural defects of HLA genes, TAPs, and β_2-microglobulin may not. Additional aberrant expression of HLA-G may enable those melanoma cells to escape also from natural killer cell immunosurveillance and provide a rationale for failure to IFNα therapy observed in these patients. These results present a technique that may help for pretherapeutic selection of patients likely not to benefit from IFNα therapy.

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An Integrated Model for the Differentiation of Chemical-Induced Allergic and Irritant Skin Reactions

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Contact and photocontact allergic as well as irritant and photoirritant skin reactions represent a major problem in clinical dermatology and the development of new pharmaceuticals. Furthermore, there is a lack of *in vitro* and *in vivo* assays that provide a clear differentiation between allergic and irritant skin reactions. Here, we describe an integrated model to differentiate between chemical induced allergic and irritant skin reactions by measuring objective and easy-to-determine parameters within both skin and skin-draining lymph nodes. Dose–response studies with standard contact and photocontact allergens as well as irritants and photoirritants revealed that irritants predominantly induced skin inflammation, which in turn stimulated draining lymph node cell proliferation. In contrast, the induction phase of contact or photocontact allergy was characterized by marginal skin inflammation, but a marked activation and proliferation of skin-draining lymph node cells. Therefore, a differentiation index (DI) was defined describing the relation between skin-draining lymph node activation (lymph node cell count index) and skin inflammation (ear swelling). A DI > 1 indicates an allergic reaction pattern whereas DI < 1 demonstrates an irritant potential of a chemical. Experiments with reference compounds such as oxazolone, TCSA ± UVA, croton oil, 8-MOP ± UVA confirmed the predictive value of DI. Furthermore, flow cytometric analysis of lymph node-derived T-and B-cell subpopulations revealed that contact sensitizer, but not irritant, induced the expression of CD69.

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Human Peripheral Blood Mononuclear Cell Response to *Propionibacterium acnes* and Heat Shock Proteins in Inflammatory Acne Vulgaris

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Propionibacterium acnes has been strongly implicated in inflammatory acne. However, its role in the disease is unclear. We have hypothesised that an immune response to *P.acnes* heat shock proteins (hsp) may play a role in the pathogenesis of inflammatory acne. This study aimed to investigate the response of peripheral blood mononuclear cells (PBMNC) from acne patients to hsp from related bacteria.

PBMNC were tested from 12 acne patients, 11 healthy controls and 10 resolved acne patients. The transformation of PBMNC to *P.acnes,* mycobacterial hsp10, 65, and 70 (0.5 μg per ml) was determined by incorporation of ^{3}H-thymidine into DNA over a nine day period. Contingency tables were used to analyse the proportion of individuals showing a significant response (LTI $\geq$ 3.0) in each group.

There was a significantly ($p < 0.05$) higher proportion of positive responders to hsp10, hsp65 and hsp70 in patients (67%, 58%, 67%, respectively), compared to controls (18%, 27%, 18%) or resolved patients (30%, 10%, 20%). There was no significant difference in the proportion of positive responders to *P.acnes* in patients (67%) compared to controls (45%) or resolved patients (80%).

These results show that a greater number of patients with active acne are sensitised to these hsp compared to controls or resolved donors. This indicated that bacterial hsp may have a role to play in the pathogenesis of inflammatory acne vulgaris.

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Enrichment and Enumeration of Wasp Venom Phospholipase Specific Memory B-Lymphocytes: No Correlation Between Serum Titer and B Cell Number

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Recent studies in mice have indicated that the longlasting specific antibody responses seen after infections, vaccinations or allergic reactions are probably due to the existence of long-lived plasma cells. Therefore, because the maintenance of humoral immunity is not necessarily dependent on continous stimulation of long-lived memory B cells, the question arises whether there is a correlation between antibody titer and memory B cell immunity. Insect venom allergy is characterized by the induction of IgE as well as IgG antibodies to different constituents of the venom. We used a new assay combining two-step immunomagnetic enrichment with multiparameter flow cytometry to detect, enumerate and characterize allergen-specific memory B-cells. To proof the specificity of the enrichment, PLA1B specific B cells were cultured and the supernatatant analyzed for phospholipase specific IgG. Here, we demonstrate using wasp venom phospholipase A1B as a major allergen in wasp venom allergic patients, that the frequencies of allergen-specific memory IgG^+ B cells and the serum titers of allergen-specific IgG in these patients do not correlate. This lack of correlation favours a model in which memory B cells and plasma cells represent independently controlled forms of immunologic memory. Furthermore this finding challenges the concept of measuring antibody titers alone to assess the persistence of humoral immunity.

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Colonisation with Superantigen-Producing *S. aureus* is associated with a More Severe Course of Atopic Dermatitis

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Atopic dermatitis is a chronic inflammatory skin disease associated with colonisation of the skin with *S. aureus* known to produce toxins with superantigen activity. Besides T cell activation these toxins induce T cell skin homing *in vitro*. This may contribute to the observed induction or enhancement of skin inflammation. The aim of this study was to determine whether colonisation with superantigen-producing *S. aureus* isolates modulates the intensity of atopic dermatitis. If so, it was of interest whether this may be preferentially due to the toxins' effects as superantigens or as allergens. In 34 AD patients, 50 healthy controls, and 21 atopic controls superantigen production by *S. aureus* isolated from skin or mucous membranes was investigated and correlated to disease severity of disease. Total IgE, superantigen-specific IgE, and T cell activation and recirculation markers were analysed and correlated with superantigen production. In 24 of 34 patients, *S. aureus* was isolated which produced superantigens in 75%. This frequency was significantly higher compared to healthy controls (33%, $p < 0.005$). Superantigen production by *S. aureus* was correlated with a significantly higher SCORAD index (53.7 ± 17.2 *vs* 42.5 ± 7.1; $p < 0.05$). However, in contrast to children with AD in adult patients ($n = 65$) the disease severity was not associated with sensitisation against the superantigens SEA and SEB. Furthermore, superantigen production by *S. aureus* was inversely correlated with total IgE concentration ($p < 0.005$) and positively correlated with T cell activation (as measured by HLA-DR and CD69 expression) and the expression of the T cell skin homing phenotype cutaneous lymphocyte-associated antigen. Superantigen production by *S. aureus* is suggested to be involved in aggravation of atopic dermatitis. Since superantigen specific IgE was not correlated with disease severity and total IgE correlated inversely with superantigen production the *superantigenic* effects (T cell activation and induction of T cell skin homing) and not the *allergenic* effects (production of total and superantigen specific IgE) of these toxins may preferentially contribute to disease aggravation.

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In Vitro Human T Cell Sensitization to TNP Using Monocyte-Derived Dendritic Cells

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The need to develop *in vitro* predictive tests which could identify potential allergens has been recognized for many years. In previous reports, we have demonstrated the ability of cultured human Langerhans cells to induce *in vitro* primary sensitization of naïve autologous T lymphocytes to strong haptens. The present study is aimed at testing the ability of human dendritic cells (DC) derived from circulating monocytes to induce *in vitro* T cell sensitization to different allergens. The allergens chosen for the study were 2,4,6-trinitrobenzene sulfonic acid (TNP), fluoresceine isothiocyanate (FITC), dinitrobenzene sulfonic acid (DNBS), eugenol and an irritant, sodium dodecyl sulfate (SDS) was used as control.

CD14+ monocytes were purified from the peripheral blood of healthy donors by magnetic depletion of T, B and natural killer cells. Monocytes were cultured for 6–8 d in the presence of GM-CSF (500 U per ml, Sandoz) and IL-4 (100 U per ml, Schering-Plough) and maturation of the cells was achieved by a further 2 d culture with TNF a. As assessed by immunofluorescence and cell cytometry analysis, the resulting cell suspensions expressed CD1a, HLA-DR, CD54, CD80, CD83 and CD86 antigens, therefore displaying a mature DC phenotype. We first analyzed the optimal conditions for *in vitro* T cell sensitization by adding a graded number of TNP-modified DC to autologous T cells. Results showed maximal T cell proliferation when 10^3 or 2×10^3 TNP-modified DC were added to 10^5 autologous T cells in a 5-d mixed lymphocyte reaction. The proliferation indexes varied according to the donors (from 3 to 30, mean $= 12 \pm 11$) but they were significant in six out of six experiments. By contrast, using similar conditions, the other sensitizing chemicals, i.e., DNBS, FITC and eugenol, as well as the irritant SLS failed to generate significant T cell proliferation. Further experiments are in progress to compare the ability of immature *versus* mature DC to induce *in vitro* T cell sensitization.

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New Insights into the Pathophysiology of Pseudoallergic Reactions

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Clinically, pseudoallergic reactions (PSAR) which are often induced by drugs such as NSAID resemble true immunologically mediated allergic reactions. Although the exact mechanisms involved in PSAR are far from being clear it is well accepted that PSAR are not mediated by specific IgE antibodies. Therefore, no reliable *in vitro* tests exist and risky and time consuming provocation tests are regarded as diagnostic gold standard. Our previous studies demonstrated that PSAR to ASA are characterized by enhanced sulfidoleukotrienes (SLT) production of IL-3 primed leukocytes suspensions after stimulation with C5a. Aim of the present study was to investigate the sensitivity and specificity of this enhanced SLT production induced by C5a and to study the effect of other basophil agonists. It was shown that not only C5a but also PAF and fMLP resulted in a significant higher production of SLT in subjects with ASA pseudoallergy proven by OPT ($n = 18$) when compared to ASA tolerant subjects ($n = 66$). Interestingly, SLT release induced by the full basophil agonist anti-FcεRI mAb was also significantly increased in patients with PSAR. However, SLT release induced by C5a, PAF or fMLP did not correlate with anti-FcεRI mAb mediated effects. As compared with OPT as gold standard sensitivity and specificity of C5a, PAF, and fMLP induced SLT release was 50–88% and 81–100%, respectively, depending on the basophil agonist used. Taken together our data indicate that PSAR to ASA are characterized by a significant SLT production in response to incomplete and full basophil agonists and that this fact can be used for reliable diagnostic tests.

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Modulation of Irritation-Induced Increase of E-Selectin mRNA *In Vivo* by Topically Applied Corticosteroids

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There is a continuous need for methods to evaluate the biological effects of topically applied drugs in the skin. Irritation of the epidermis with sodiumdodecylsulphate (SDS) leads to an upregulation of E-selectin on endothelial cells and E-selectin mRNA can be detected *in vivo* within short time. We investigated whether this biological response can be used as a read-out for the anti-inflammatory effect of topically administered corticosteroids with different strength. On the skin of healthy volunteers, two different protocols were performed: (1) topical application of different corticosteroids (*versus* basic ointments as controls) for 12 h and irritation with SDS 1% for 4 h, (2) irritation with SDS 1% for 12 h and application of the corticosteroids for 5 h. The biopsy specimens were subjected to RNA extraction and reverse transcription and competitive RT-PCR was performed using defined concentrations of a preconstructed mimic DNA. As result, we found strong positive signals for wild type E-selectin mRNA in all biopsies pretreated with basic ointments, whereas in biopsies from areas pretreated with corticosteroids the bands for wild-type E-selectin DNA could be detected at 10–1000 lower levels of mimic DNA concentrations. The application of corticosteroids after irritation for 12 h again yielded significantly reduced signals for E-selectin mRNA found in the biopsies. Our study demonstrates the pharmacological effect of topical corticosteroids on the irritation-induced E-selectin mRNA expression on dermal endothelial cells *in vivo* using very small tissue samples and our approach may be of value for further pharmaceutical studies.

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Phenotypic Characterization of T Cells in Human Afferent Lymph From Normal Skin

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T cells represent an important cell population of the skin associated lymphoid tissue. The aim of this study was to analyze and compare the phenotype of T cells with regard to their surface molecules and cytokine expression in the afferent human lymph and peripheral blood. By means of microsurgical lymph cannulation afferent lymph derived from normal skin was obtained from 10 volunteers. Lymph cells were isolated and expression of CD3, CD4, CD8, αβTCR, γδTCR, HLA-DR, CD11a, CD18, CD19, CD25, CD26, CD28, CD30, CD45RO, CD45RA, CD54, CD56, CD69, CD71, CD62L, CD154/40L, CCR3, CCR5 as well as intracellular INF-γ, IL-4, IL-5, IL-10 was analyzed by flow cytometry. Furthermore, the expression of IL-12 was investigated by immunohistochemistry. The majority of the lymphoid cell population in the afferent lymph were T cells and belonged to the lineage expressing the αβTCR. The CD4:CD8 ratio in the afferent lymph ranged from 2:1 to 4:1. In contrast to the peripheral blood, expression of CD45RO was found on most of the $CD4^+$ and $CD8^+$ cells, indicating that these cells are mainly memory/effector T cells. An increased percentage of these cells expressed activation molecules like HLA-DR, CD25, CD26, CD69 as well as adhesion and costimulatory molecules like CD54, CD154/40L. T cells in the afferent lymph predominantly expressed surface molecules such as CD26 and CCR5, which have preferentially been reported on T cells producing type 1 cytokines. In addition, $22.2 \pm 7.9\%$ (mean ± SD) of the T cells in the afferent lymph stained for the type 1 cytokine INF-γ, whereas type 2 cytokines such as IL-4, IL-5, IL-10 were not or barely detectable. Interestingly, dendritic cells expressing IL-12 were also found in close association with some lymphocytes. Our data demonstrate that memory T cells, expressing increased levels of activation, adhesion and costimulatory molecules as well as a type 1 cytokine profile migrate through afferent lymph derived from normal skin in humans and suggest that these T cells, favoring a cell-mediated immune response, are an important part of the immune surveillance against pathogens or other antigens in the skin and its associated lymphoid tissue.

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Mast Cell Precursors in the Peripheral Blood of Patients with Atopic Diseases and Psoriasis

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Increased mast cell numbers are observed in the tissue of patients with various allergic and certain chronic inflammatory diseases, but the mechanisms involved and the nature of the mast cell progenitors are still poorly understood. We have previously shown that cells from the adherent mononuclear fraction of peripheral blood are able to differentiate towards mast cells. These cells were therefore obtained from normal donors ($N = 8$) and patients with atopic dermatitis or rhinitis ($N = 8$), psoriasis ($N = 8$) and non-IgE-related intrinsic asthma ($N = 3$). Expression of the FcεRI-α, β, γ-chain, the mast cell differentiating factor SCF, the SCF-receptor c-Kit and histidine decarboxylase were examined by semiquantitative RT-PCR and immunocytochemistry. FcεRIα, SCF and histidine decarboxylase were increased in atopic donors and patients with psoriasis, compared to normal donors, whereas FcεRI-β and c-Kit were only increased in atopic donors and FcεRI-γ only in patients with psoriasis. No differences were found in patients with intrinsic asthma, compared to normal controls. These data show thus that in patients with allergic diseases and increased tissue mast cell numbers, increased numbers of cells with characteristics of immature mast cells are present in the peripheral blood mononuclear fraction. The nature of these cells as well as that of the SCF and FcεRI-α expressing cells will have to be further clarified, also at the protein level.

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Flowcytometric Characterisation of T-Cells in CTCL Lesions

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Indolent, primary cutaneous T-cell lymphomas (CTCL) are characterised by clonal proliferation of malignant T-helper cells in the skin as well as reactive T-cell infiltration. Using quadruple color flow cytometry after mechanical disaggregation (without enzymatic digestion), we examined the expression (%) of activation antigens as well as the cytokine production of infiltrating T-cells in skin samples (patch and plaque) from 11 CTCL patients. The T-cell immunophenotype in CTCL-involved skin can be summarised as mainly activated memory/effector T-cells (CD4+ or CD8+): CDII a high, CLA+, CD45RO+, with a majority expressing the activation antigens HLA-DR, CD25 (IL-2Ra), or CD69. Interestingly, the expression of these markers was higher in the CD4+ than in the CD8+ T-cell subset. Using PMA/Ionomycin stimulation and Monensin to achieve short-term intracellular cytokine accumulation, we found that T-cells express IFN-g (22%), TNF-a (17%), IL-2 (10%), and IL-4 (15%). In two cases of advanced stage CTCL we were able to characterise the malignant TCR-VBX+ T-cells. In a patient with tumour stage of mycosis fungoides we demonstrated the malignant T-cell as TCR-Vb 3+, CD4+, CD45RO+, and HLA-DR+. Interestingly, a completely different pattern was found in an other tumour from CTCL patient (TCR-Vb22+). The striking finding here was the lack of memory markers as well as of CLA and HLA-DR expression on the malignant cells. In contrast to infiltrating T-cells, TCR-Vb22+ T-cells exhibited almost no PMA-stimulated cytokine production. In summary flow cytometry of lesional dermal tissue represent a useful new opportunity for investigations of T-cell subpopulation in CTCL and other T-cell mediated dermatoses.

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Molecular Analysis of TNF Alpha and Beta Genes in Patients with Multiple Non Melanoma Skin Cancer

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Exposure of the skin to Ultraviolet B (UVB) radiation cause an immunosuppression which is thought to be a contributing factor to the induction of skin cancer. Several mechanisms have been implicated in UV-induced immunosuppression, including the synthesis and release of immunoregulatory molecules and cytokines. Contact Hypersensitivity (CH) model to DNFB demonstrated in mice that: (i) TNF-alpha is a major mediator of UVB-induced immunosuppression; (ii) susceptibility to UVB-induced immunosuppression is genetically determined: strains that fail to develop CH to DNFB after UVB irradiation are termed UVB-susceptible (UVB-S) while strains that are impervious to this effect are termed UVB-resistant (UVB-R). Similar studies in humans have demonstrated that the UVB-S and the UVB-R phenotypes also exist in humans and that virtually all the patients with a biopsy proven skin cancer are UVB-S. Genetic studies in mice have revealed that UVB-susceptibility is a polygenic trait. Particularly, polymorphisms within TNF locus are considered to be relevant in governing UVB-susceptibility, probably by controlling TNA-alpha production at transcriptional and/or translational level. In order to study a possible role of TNF-alpha and beta genes in determining a predisposition to multiple UV-induced skin cancer, two polymorphisms in the TNF region have been analyzed: the microsatellites TNFa e TNFb. Forty patients affected with multiple non melanoma skin cancer (basal and squamous cell carcinomas) and 111 Italian healthy controls have been studied. A statistically significant association has been found between the TNFa6 allele and the patients (R.R. = 3.5; $Pc < 0.025$). This result suggests that in the TNF region a gene is located that could play a role in predisposition to multiple skin carcinomas.

Since for ethical reasons we did not perform CH studies in our patients, we can only speculate that TNF alpha gene modulates the UV-induced immunosuppression and consequently the susceptibility to skin cancer.

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The Cholinergic System in Palmar Skin of Healthy Subjects and Patients with Palmo-Plantar Pustulosis

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Ninety-five percentage of PPP patients are smokers. We have recently demonstrated that the acrosyringium is the site of inflammation in PPP. The sympathetic fibers that innervate the sweat glands are cholinergic. Acetylcholine (ACh) has an effect on sweating via the acetylcholine receptor (AChR). Two types of acetylcholine receptors have been identified, the nicotinic (nAChR) and the muscarinic (mAChR). Nicotine acts as an agonist on nAChR, and smokers are known to have an increase in sweat production.

The aim of this study was to investigate with immunohistochemistry the distribution of choline acetyltransferase (ChAT), acetylcholinesterase (AChE) and nicotinic receptors in palmar skin in healthy nonsmoking/smoking subjects and in patients with palmo-plantar pustulosis (PPP).

ChAT and AChE were strongly expressed in the eccrine gland and duct in all subjects. In smokers the ChAT reactivity was significantly decreased. In PPP patients the distribution in the eccine gland and duct was similar to that in smokers. In PPP a strong ChAT reactivity was also displayed in the neutrophil and eosinophil granulocytes migrating into the pustule. The number of mast cells was highly incresed and 30% were AChE positive. The presence of ChAT-like proteins in neutrophils and eosinophils was confirmed by Western blot analysis. In neutrophils, ChAT-like proteins with different molecular weights were found, one band at 54 kDa and also a dimer at about 69 kDa, whereas in eosinophils and placenta (used as a positive control) one prominent band was seen, with a molecular weight of 54 kDa.

Immunohistochemistry indicated the presence of α-3, α-4 and α-7 subunits of the nicotinic receptors in palmar skin.

The present results indicate that the cholinergic system may be involved in the inflammatory process in a hitherto unknown way.

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Different Pathogenesis of T-Cell Reactions to Paraphenylenediamine and its Autoxidation Product Bandrowski's Base

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Allergies to low molecular weight compounds are of major public and occupational importance. Paraphenylenediamine (PPD) is one of the most common allergens among patients with allergic contact dermatitis. However, little is known about the nominative antigens which induce T-cell proliferative responses, except for a possible role of Bandrowskiś Base (BB). Therefore, we investigated the immunogenic capacities of these compounds and BB by measuring the proliferative responses of T-cells. Proliferative responses were observed to PPD and BB (SI = 2.9–40.0). T-cell clones were established reacting to PPD as well as to BB. FACS-analysis revealed that all clones were CD45 RO and CD4 positive except for one CD8 positive clone. All clones expressed TCR-$\alpha\beta$. T-cell clones released high amounts of IL-5 (10–30 ng per ml) and lower amounts of IFN-γ (0.5–3.0 ng per ml). The MHC restriction was evaluated by inhibition using anticlass II mAb. Proliferation assays in the presence of mAb revealed a strong inhibition using HLA-DP (88–97%), whereas inhibition of T-cell responses by anti-HLA-DR and anti-HLA-DQ antibodies was less pronounced (11–27%). To determine whether processing is required for PPD and BB recognition by T-cells, glutaraldehyde-fixed APCs were tested for their ability to stimulate T-cell clones. T-cell proliferation to BB was strongly inhibited (70–95%) in BB specific T-cell clones using fixed APCs whereas T-cell reactivity to PPD was not affected in all but one PPD specific T-cell clones. Continuous presence of PPD was required during the assay period because pulsing of the APC was not sufficient to induce T-cell proliferation. This was not the case for BB. The APC could be fixed without impairing their ability to present BB. In summary, the established T-cell clones reacted to BB as well as to PPD. However, our data suggest that the pathogenesis of T-cell reactions to PPD and BB may be different. PPD can be presented in a MHC restricted fashion, which is independant of processing, whereas, T-cell responses to BB were strictly dependent on processing of the allergen. This study was supported by EU-PL 963713.

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Expression of E-Selectin is Mandatory for Vasculitis as are Fixed Immune Complexes, but its Function is Beyond Recruitment of PMN

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We have shown that injection (a) of immune complexes (Arthus reaction), (b) of repetitive doses of LPS and (c) of sphingomyelinase-containing spider venom into mice all result in a histological picture consistent with leukocytoclastic vasculitis (LcV). In the LPS-elicited, Shwartzman-like LcV sustained expression of adhesion molecules is one characteristic feature. In humans, however, immune-complexes (IC) are the most frequent cause for LcV, albeit we will show that their vascular deposition does not always result in LcV. We therefore wondered (1) how IC lead to vessel damage, (2) if adhesion molecules play a critical role also in IC-mediated LcV.

In order to imitate the effects of vascular deposition of IC we fixed large IC (made of equimolar concentrations of HSA and murine anti-HSA) to nitrocellulose, added PMN (murine or human) and measured degranulation (β-glucuronidase/LDH). Fixed, large IC caused significantly higher degranulation than the same concentration of large IC kept in solution or than small IC (made of Ag >> Ab). Thus, vascular fixation of IC could promote damage to endothelial cells and LcV by causing release of proteases at the vessel wall.

However, when screening human dermatoses for vascular deposits of Ig we found vascular Ig also in nonvasculitic dermatoses (e.g., 30% of porphyria cutanea tarda and 10% of lichen planus) as well as in *un*involved skin of patients with LcV and in mice after iv injection of IC. Thus, vascular deposits of Ig are not sufficient to cause LcV. In all these biopsies we also did not find a strong expression of E-selectin which, however, we did in LcV. Thus, we analysed the role of CD62E *in vivo* by injecting neutralizing Ab to CD62E in the murine Arthus reaction (Art-r). It significantly reduced hemorrhage in the Art-r, while it did not change the size of the infiltrate.

Thus, IC cause vigorous degranulation of PMN when they are large and fixed, but *in vivo* their vascular deposition needs to be combined with the expression of E-selectin to cause LcV. As neutralization of E-selectin did not impair the recruitment of PMN, E-selectin must have additional effects, e.g., such as activation of PMN.

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Engagement of ICAM1 and HLA-DR by Staphylococcal Superantigen on Depleted Stratum Corneum Intercellular Lipid Lamellae: Protection by Emollient Preparation

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The stratum corneum (SC), an heterogeneous protective layer, with a protein fraction included in a lipid matrix, represents the skin barrier function. The hydrophobic lipids are sequestered as multilayered lamellae within the intercellular spaces and regulate transepidermal water loss, corneocyte cohesion and percutaneous penetration.

In this study, we have demonstrated that a single topical application of Staphylococcal Enterotoxin B Superantigen (SEB) on a barrier-disrupted skin, initiated stronger reponses than when applied on a non lesional skin. This alteration was realised by a solvent treatment on a skin biopsy. The epidermal disorganisation and the restructural effect of an emollient substance application were shown by various morphometric analysis; layers of the SC were evaluated by methylene blue stain after alkaline expansion; total and neutral lipids were stained by black Soudan B and oil red O. Epidermal and dermal dendritic antigen-presenting cells are the main targets of SEB. Their functional activation or changes are accompanied by specific immunophenotypic modifications or alterations: high CD1a+, HLA-DR+ and expression of costimulatory adhesion molecules such as intercellular adhesion molecule ICAM1, required for T-cell activation. Immunohistochemical studies indicate that SEB exerts a diverse range of effects on a multiplicity of cutaneous cell types, both under basal conditions and acute barrier perturbations. The emollient preparation (Triχera), diffusing through the SC interstices, restore normal barrier function, and inhibits the SEB penetration and its potential involvement in the cutaneous inflammatory diseases.

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Evaluation of Natural Killer Cell Activity in Patients Affected with Photoinduced Herpes Simplex

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It is well known that exposure to UV light can induce an immunosuppression interfering at different levels of the immune response. UV exposure may affect the immune response to microbial infections that could result in increased incidence and severity of the disease, or in an altered outcome in the balance between the microorganism and the host in the case of persistent infections.

A documented instance where UV affects a microbial infection in the natural host is labial herpes simplex in humans. In fact the virus which is present in the latent form after the primary infection, can be reactivated provoking clinical manifestation after a sun exposure. Most of the effector arms of the immune system are involved in the control of HSV infections although cell mediated mechanisms are considered the most effective. In particular natural killer cells seem to have a crucial role in the defense from infections.

In the present study we have evaluated natural killer cells lytic activity in 10 patients affected with labial herpes simplex and 10 sex and age matched controls.

The peripheral blood lymphocytes (PBL) samples were collected early in the recurrence (1° or 2° day of the disease). In all the patients studied the recurrence was induced by a exposure to erythemal doses of sunlight. In five patients we have also taken samples of PBL during the latency. In both patients and controls we have also evaluated lymphocytic production of IL-2, IL-4, IFNγ.

Our results show that early during the recurrence induced by an exposure to sunlight natural killer cells lytic activity is significantly reduced in comparison with controls. Furthermore, in the same patient, natural killer activity during the recurrence is significantly decreased in comparison with the latency.

There is no significant difference in natural killer activity between patients during the latency and controls.

No significant difference was found in lymphocyte IL-2, IL-4 and IFNγ production.

In conclusion we can hypothesize that sun exposure could induce a decrease of natural killer activity that contribute to an imbalance between virus and host that leads to the development of the disease.

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Histological and Immunohistochemical Study of the Skin of the First Human Hand Allograft

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In September 1998, the first human hand allograft was successfully performed in our hospital. The recipient, a 48-y-old white man, received a hand allograft from a 42-y-old white man brain dead from intracerebral hematoma; the donor and recipient shared the same blood group but had six HLA mismatches. After grafting, the recipient was treated with FK506, antithymocyte globulins, mycophenolate mophetil and prednisone. Sequential skin biopsies were taken from the grafted hand at various days postgraft (d5-d103) and examined (immuno)histologically in order to assess the quality of the grafted skin and to monitor a possible graft rejection. Histologically, the skin appeared normal, with the exception of days 57 and 63, when a rather dense mononuclear cell infiltrate of recipient's origin (HLA-A24+) was seen in the dermis. This had appeared concomitantly with erythematous, infiltrated skin lesions that developed after a decrease of FK506 dosage, and were considered to represent signs of graft rejection. This was reversed with an increase of the immunosuppressive treatment. No skin necrosis was seen at any time, and on the latest biopsies (d85) the inflammatory changes had almost disappeared. Immunohistochemically, the main cell types of the skin (epidermal Langerhans cells, melanocytes, dermal dendrocytes, endothelial cells, Schwann and smooth muscle cells) were present throughout the study period, and expressed their characteristic immunohistochemical features. The density of Langerhans cells was normal, and from day 77 the epidermis of the grafted hand harbored some epidermal Langerhans cells of recipient's origin (CD1a+/HLA-A24+). There results show that in this composite tissue graft, skin biopsies may reveal a rejection of the graft; it also shows that the allografted skin maintains a normal structure and contains all essential cell types, including resident cells of recipient origin, such as Langerhans cells.

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Is there a Mitogen or Superantigen produced by Propionibacterium acnes?

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Propionibacterium acnes is a major stimulus in acne, either initiating or maintaining the disease. Mitogenicity of *Corynebacterium parvum,* now named *P. acnes*, has been reported. Therefore, a lymphocyte stimulation assay was carried out with seven fresh bacterial isolates of *P. acnes* from patients with different stages of inflammatory acne and the laboratory strain *P. acnes P 37*. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of 9 healthy volunteers and 11 new borns by density gradient centrifugation. The PBMCs (10^5 per 100 μl) were cocultured either with a mixture of *P. acnes* strains at different concentrations or RPMI culture medium alone for various periods of time. The mitogen phytohemagglutinin (PHA) and the superantigen TSST-1 served as positive controls. Cell proliferation was measured by adding 0.25 μCi per well [6–^{3}H]Thymidine (Amersham, Little Chalfont, U.K.) to PBMCs during the final 4 h of the incubation period. Eight of 11 cord blood donors showed significantly positive reactions (stimulation index (SI) $>$ 3), two of eight nearly reacted as high to *P. acnes* as to the mitogen PHA. Five of nine healthy adults showed reactivity (SI ranged from 3.7 to 31.0). These results demonstrate that there is a response of adult as well as naive PBMCs to *P. acnes* which is not mitogenic but in some cases too strong to be considered as antigenic. Superantigenicity may be the explanation.

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Systemic Provocation in Contact Allergy to Gold – An Experimental Model for Flare Up Reactions

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Contact allergy to gold was diagnosed in 35 patients with eczematous disease by patch testing with a standard series containing gold sodium thiosulfate and then confirmed by a serial dilution test. All patients were given one intramuscular injection with gold sodium thiomalate. Clinical reactions comprised an eczematous flare up of previously positive patch tests to gold in 80% and this was specifically induced; a flare up of a previous dermatitis in 25%; a toxicoderma-like rash in 46%; and a transient fever in 60%. The flare up of patch tests implied a vigorous increase of cutaneous blood flow visuallized by laser Doppler perfusion imaging within 1 h after systemic provocation. Tissue priming in these old patch tests seems to comprise memory T-cells and ELAM-1 in upper dermis; in the reactivation process, mast cells as well as blood-borne monocytes seem to play essential roles. Concomitantly with the clinical and immunohistochemical flare up there is a release in peripheral blood of cytokines and acute phase reactants.

Parenteral provocation in contact allergy to gold provides an easily monitored, experimental human model for studying the pathogenesis of endogenous contact dermatitis with special reference to clinical, biochemical and immunohistochemical aspects of the flare up mechanism.

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Are Epidermal $CD8^+$ T Cells in Chronic Plaque Psoriasis Specific for Group A Streptococcal Antigens?

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Epidermal $CD8^+$ T cells in chronic plaque psoriasis show oligoclonal expansion suggesting antigen specificity; the antigen involved is, however, unknown. The aim of this study was to determine whether, in common with dermal $CD4^+$ T cells in psoriatic skin lesions, epidermal $CD8^+$ T cells proliferate in response to group A streptococcal antigens. Epidermal $CD8^+$ T cells were isolated from trypsinized epidermal cell suspensions from lesional shave biopsies of six patients with chronic plaque psoriasis using an anti-CD8 monoclonal antibody and magnetic beads. The T cell lines (TCL) were cultured with sonicated, heat-killed group A streptococci (Strep-A) and IL-2, and tested in proliferation assays with Strep-A or medium, with or without IL-2.

Four epidermal TCL were obtained, three of which were 67–99% $CD8^+$; the fourth TCL was 98% $CD4^+$. The three $CD8^+$ TCL proliferated to IL-2, but not to Strep-A alone; furthermore, no increase in proliferation was observed when Strep-A was added to IL-2. In contrast, the $CD4^+$ TCL responded strongly to Strep-A in the absence of IL-2, and to IL-2 alone.

Thus epidermal $CD8^+$ TCL from chronic plaque psoriasis lesions are not reactive with streptococcal antigens as assessed by proliferation. Other responses to
Strep-A such as cytokine production are being investigated.

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Apoptosis in Cutaneous Lupus Erythematosus – A Histochemical Study

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Dysregulation of apoptosis has been shown to play a role in the development of autoimmune diseases such as lupus erythematosus (LE). In the current study lesional skin cryostat samples from 30 patients with cutaneous manifestations of LE (17 DLE, 10 SCLE and three SLE) were stained immunohistochemically with monoclonal antibodies to Fas antigen, Fas ligand (FasL), Bcl-2 and Bax proteins and apoptotic cells were detected with TdT-mediated dUTP-biotin nick end labeling. The basal cells of the lesional epidermis showed a marked increase of Fas expression while the expression of Bcl-2 was distinctly decreased. There was also an increase of FasL and Bax positive cells within the lymphohistiocytic infiltrate in the dermis but not in the epidermis or in the skin appendages in comparison to normal skin (n = 10). With the TUNEL method it was demonstrated that extensive apoptosis occurred in the epidermis, among the cells of the dermal infiltrate, and sporadically also within the hair follicles in LE skin.

In the present study diverse markers of apoptosis have been studied comprehensively. This included the investigation of Bax protein expression in LE skin for the first time. This study proved the involvement of the apoptotic process in the pathogenesis of cutaneous manifestations in lupus erythematosus.

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In Vitro Anti-Radical Effect Evaluation of *Avena Rhealba*

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During cellular metabolism, inflammatory process or pathogenesis of human diseases, oxygen radicals are generated in response to stress. The overproduction of reactive oxygen species induces many alterations damaging especially cellular targets such as the plasmic membrane components, the proteins and the genomic material. In the skin, the stress consequence is expressed by an acute inflammation, a tissue aging and sometimes by an initiation of tumor process.

In order to evaluate the effect of *Avena Rhealba* extracts on inflammation and more especially on oxygen radicals generation, we have investigated their protective efficiency on the main and first target of the oxidative stress, the cellular plasmic membrane. We have induced cellular oxidative stress, signal of inflammation, by treating human cutaneaous fibroblasts with the complex H_2O_2-Fe^{2+}/Fe^{3+}. Like this, by reproducing the Fenton reaction and an overproduction of hydroxyl radical (OH°), we have quantified the lipoperoxidation level using the TBARS Assay, which measures the ThioBarbituric Acid Reactive Substances generated between thiobarbituric acid and oxidized aldehyde (like the malondialdehyde), and we have evaluated the modulation of this lipoperoxydation by *Avena Rhealba* extracts increasing doses.

Results from these *in vitro* studies clearly show that *Avena Rhealba* extracts limit the damaging activation of reactive oxygen species on the lipid component of the plasmic membrane. In conclusion, by its antioxidant activity, *Avena Rhealba* preserves the integrity of the cutaneous tissue from dramatical effect of free radicals on *stratum corneum* lipids during inflammation process of the skin, particularly atopic dermatitis.

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Extrarenal Manifestations of Recurrent Wegener's Granulomatosis in a Renal Transplant Recipient

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Wegener's granulomatosis (WG) is a systemic necrotizing vasculitis of small vessels, predominantly affecting kidneys and the lung. Cutaneous manifestations are rare and present as hemorrhagic necrotic papules and blisters. Treatment of choice is a combination of cyclophosphamide and prednisolone. We report a 71-y-old patient who underwent renal transplantation due to progressive glomerulonephritis in 1990. Her immunosuppressive regimen included cyclosporine A and corticosteroids. Eight years later, she experienced severe arthralgia including ankle, wrist, knee and elbow joints. On laboratory examination, an elevated ESR (60 mm per h), a WBC of 15.6×10^9 per liter with 21% eosinophils and positive cANCA (1:1024) were noted. In parallel, she developed hemorrhagic blisters and necrotizing papules on both ventral and dorsal aspects of her feet and on her fingertips. Upon biopsy, a necrotizing vasculitis involving small superficial arteries and venules was apparent as judged by staining with CD31. By staining with CD68 and CD34, a few granulomas were seen in the deeper parts of the dermis. Notably, eosinophils were scattered throughout the infiltrate. The involvement of small arteries and venules and the presence of cANCA argue for the diagnosis of Wegener's granulomatosis as opposed to polyarteriitis nodosa. Although a marked eosinophilia was present in the lesions and the peripheral blood, she had no history of allergic rhinitis or asthma. Also, serum IgE was normal, both arguing against Churg–Strauss syndrome (CSS). Three days after the initiation of cyclophosphamide/prednisolone therapy, she developed fulminant alveolar hemorrhage and died of respiratory failure.

In conclusion, a fatal recurrence of a distinct WG variant (with eosinophilia) may occur despite immunosuppressive therapy. It is important to note that extrarenal such as cutaneous manifestations may be early signs of a relapse of this disease.

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Increased Numbers of Apoptotic Keratinocytes in Human Epidermis of Cutaneous Lupus Erythematosus

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Apoptosis is known as a process of programmed cell death that has been suggested to be an important mechanism for the expression of autoantigens on the surface of human epidermal keratinocytes. In lupus erythematosus (LE), a prototype of an autoimmune disease with different cutaneous manifestations, antibody-dependent cellular cytotoxicity is regarded as a major pathological mechanism. However, the pathomechanism underlying cutaneous LE is incompletely understood and therefore requires further investigations. To address whether increased apoptosis may contribute to the pathogenesis of cutaneous LE skin biopsies were taken from 50 patients with different subtypes of LE. Using DNA-polymerase for *in situ* nick translation (ISNT) and terminal deoxynucleotidyl transferase for end labeling (TUNEL) we investigated primary (n = 20) and UV-induced (n = 30) skin specimens of LE patients. In both, primary and UV-induced lesions of cutaneous LE, the mean rate of apoptotic cells in the granular layer of the epidermis was found to be significantly increased compared to healthy controls (n = 10). No differences in the rate of apoptotic cells were detected with respect to the different clinical subtypes of cutaneous LE. We further investigated skin specimens of 20 patients with different subtypes of LE taken 24 and 72 h after a single dose of UVA (340–400 nm) or UVB (285–350 nm) irradiation and found an increase of apoptotic nuclei throughout the whole epidermal and upper dermal layers for a prolonged time as compared to healthy controls (n = 10). These results suggest an impaired induction of the apoptotic pathway in patients with cutaneous LE which might play a role in the pathogenesis of primary and UV-induced LE skin lesions.

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Two New α-Melanocyte Stimulating Hormone (α-MSH) Analogues (MS05 and MS09) are Potent Immunomodulators *In Vivo* and *In Vitro*

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The Proopiomelanocortin derived neuropeptide α-MSH recently has been shown to be a potent immunomodulator. Among many local and systemic effects α-MSH is an antagonist for IL-1, as well as TNFα and is able to suppress contact hypersensitivity and to induce hapten-specific tolerance in mice. Since α-MSH is not toxic and its use in humans did not have deleterious effects, it offers a great therapeutic potential. A disadvantage is its limited stability and rapid degradation by proteases. Therefore, stable α-MSH analogues (MS05, MS09) were synthesised and tested for their efficiency. Human dermal microvascular endothelial cells were treated with TNFα (250 μ per ml) and MS05 or MS09 (10^{-6}–10^{-12} M) and tested for expression of E-Selectin, VCAM and ICAM mRNA and protein using RT-PCR and flow cytometry. Furthermore the activation of nuclear factor κB (NFκB) was assessed by EMSA. Expression of adhesion molecule mRNA was significantly reduced by both peptides and a downregulation of NFκB activation was observed. The *in vivo* efficacy of the peptides on CHS-reaction and tolerance induction in mice was tested. Both peptides suppressed the DNFB-induced CHS-reaction and induced hapten-specific tolerance. These data for the first time demonstrate the anti-inflammatory potential of newly designed α-MSH analogues with improved stability. Due to their low toxicity and high tolerability they may turn out as a new class of potent anti-inflammatory drugs.

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IL-17 is Produced by Nickel-Specific Th1, Th2 and Th0 cells, and Cooperates with IFN-γ and IL-4 in Amplifying Inflammatory Responses by Human Keratinocytes

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IL-17 is a T cell derived cytokine detected in allergic contact dermatitis (ACD) skin, and previously shown to augment IFN-γ-induced expression of ICAM-1 and to regulate IL-8 and RANTES production by keratinocytes. In this study, we have analyzed whether IL-17 production segregate with a particular nickel-specific Th cell susbset. In addition, we examined the capacity of IL-17 to modulate the immune activation of keratinocytes induced by Th1 and Th2 cytokines. A panel (*n.* 83) of nickel-specific $CD4^+$ T cell clones were isolated from peripheral blood or ACD skin, and stimulated with PMA and ionomycin or anti-CD3/anti-CD28 mAbs. IL-17 was produced (0.1–2.5 ng per 10^6 cells per 24 h) by a portion of Th0 (16 of 37, 43%), Th1 (17 of 32, 53%) and Th2 (six of 14, 43%) as assessed by ELISA. IFN-γ-induced expression of ICAM-1 on keratinocytes was strongly and selectively enhanced by IL-17 and/or IL-4 treatment (both at 50 ng per ml). IL-17 alone, and more efficiently together with IFN-γ and/or IL-4, also stimulated keratinocytes to release GM-CSF and IL-6. In addition, IL-17 cooperated with IFN-γ and/or IL-4 in decreasing the IL-1RA/IL-1α ratio in the supernatants as well as in cell lysates from cultured keratinocytes. In contrast, IL-17 had no or very limited effects on SCF production by keratinocytes. Taken together, the results suggest that IL-17 is an important player of T cell mediated skin immune responses by amplifying IFN-γ- and IL-4-induced immune activation of keratinocytes.

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Epitope Mapping of Tyrosinase with Sera from Patients with Vitiligo

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The aim of the present study was to identify the B cell epitopes on the melanogenic enzyme tyrosinase which are recognised by tyrosinase autoantibodies in a subset of patients with vitiligo. Deletion derivatives of tyrosinase cDNA were constructed and then translated *in vitro* with the concomitant incorporation of [^{35}S]methionine into the protein products. The ^{35}S-labelled tyrosinase derivatives were then used in radioimmunoassays to investigate the reactivity of sera from five vitiligo patients and from 20 healthy individuals. Briefly, sera were incubated with each radiolabelled ligand, protein G Sepharose beads added in order to bind IgG-ligand complexes and the immunoprecipitated radioactivity evaluated in a scintillation analyser. Each serum was tested three times in each assay and the mean counts per minute immunoprecipitated were determined. Sera were considered positive if their antibody reactivity to a particular tyrosinase derivative was greater than the mean reactivity + 3SD of a population of 20 controls.

The epitope regions identified were: three in a central region of tyrosinase (amino acids 240–255, 289–294 and 295–300) and two others towards the C-terminal end of the protein (amino acids 435–447 and 461–479). Computer analysis of the potential B cell epitopes on tyrosinase revealed that the epitope regions recognised by the vitiligo sera were located in areas predicted to be highly antigenic. In addition, the centrally located antigenic regions (amino acids 289–294 and 295–300) had amino acid sequence homology to both tyrosinase-related protein-1 and tyrosinase-related protein-2.

Thus, the epitopes on tyrosinase recognised by vitiligo patient sera are heterogeneous and include a region with homology to two related proteins suggesting that the humoral response against tyrosinase in some vitiligo patients may in part result from immunological cross-reactivity between melanogenic proteins.

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IL-4 Enhances IFN-γ-Induced Expression of IP-10, Mig and I-TAC Chemokines in Cultured Human Keratinocytes

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IP-10, Mig and I-TAC belong to the non-ELR CXC chemokine family and act solely through the CXCR3 receptor for potent attraction of T lymphocytes. IFN-γ-stimulated keratinocytes secrete high levels of IP-10 *in vitro* and appear to be the major source of IP-10 in various skin disorders, including allergic contact dermatitis (ACD) to nickel. In this study, we evaluated the capacity of the T cell derived cytokines IL-17, IL-4 and IL-10 to modulate IFN-γ-induced IP-10, Mig and I-TAC by cultured human keratinocytes, and CXCR3 distribution in skin affected by ACD as well as in skin-and blood-derived nickel-specific $CD4^+$ T cell lines. IL-4, but not IL-10 or IL-17 (all at 50 ng per ml), significantly increased IFN-γ-induced release of IP-10 and Mig by keratinocytes, as assessed by ELISA. This enhancing effect of IL-4 on IP-10 release was evident after 8 h and peaked at 24 h after IFN-γ stimulation, whereas Mig superinduction was delayed at 24–48 h. IFN-γ activated keratinocytes were also found to accumulate very high amounts of I-TAC transcripts in a time-dependent manner, reaching a peak of expression at 24 h. Likewise IP-10 and Mig, I-TAC was induced to higher levels when IL-4/IFN-γ cotreatment was performed. Immunohistochemical analysis of biopsies from 24 to 48 h positive patch tests to nickel revealed that > 80% of infiltrating cells were reactive for CXCR3. Resting nickel-specific $CD4^+$ T cell lines established from blood or diseased skin were also strongly positive for CXCR3. Upon activation with anti-CD3 mAb, CXC3 levels promptly decreased, remained low at 24 and 48 h, and then returned high after 96 h. In conclusion, IL-4 exerts a relevant pro-inflammatory function by potentiating IFN-γ-induced IP-10, Mig and I-TAC keratinocyte expression which in turn may determine a massive recruitment of resting $CXCR3^+$ T lymphocytes at inflammatory reaction sites.

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IL-1α Induces Keratinocyte Hyperproliferation in Raft Culture via IL-8 and GROα Induction

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Psoriasis is a chronic inflammatory disease with an increased mitotic rate of basal and suprabasal keratinocytes as a prominent feature. IL-8 and GROα have been found to be highly expressed in psoriatic epidermis. Both chemokines are known to be potent neutrophil activators and were discussed as potential keratinocyte growth stimulators.

We therefore examined the action of GROα and IL-8 in organotypic raft culture. Both IL-8 and GROα lead to a time- and dose-dependent epidermal hyperproliferation in organotypic culture. In cryostat sections an increased number of epidermal layers as well as a significantly elevated number of Ki-67 positively stained keratinocytes indicate marked hyperproliferation with no evidence for the reduction of apoptotic cells. Differentiation was shown to be regular by the formation of a cornified layer and the expression of suprabasal keratines as well as markers of the terminal differentiation.

IL-8 mediated proliferation was inhibited by a blocking humanized monoclonal antibody, but not by an equal concentration of a matched nonspecific control antibody. To demonstrate a specific receptor mediated action of GROα and IL-8, we used a CXC receptor 2 selective nonpeptide receptor antagonist, which leads to inhibition of IL-8 mediated hyperproliferation. The CXCR2 is known to bind both chemokines with nearly the same affinity and has been shown to be highly overexpressed in lesional psoriatic keratinocytes.

IL-1 is described as inducer of IL-8 and GROα and has been shown by several groups to be over-expressed in psoriatic keratinocytes. When examined in our organotypic culture, IL-1α causes induction of IL-8 and GROα mRNA as well as marked epidermal hyperproliferation. The IL-1α mediated hyperproliferation was strongly reduced by both, the IL-8 specific antibody and the CXCR2 selective receptor antagonist, which indicates a close correlation between the IL-8/CXCR2 pathway and IL-1 induced keratinocyte growth stimulation. Our data suggest that the IL-1 induced overexpression of IL-8 and GROα could lead to the characteristic epidermal changes observed in psoriasis as well as other chronic inflammatory diseases and possibly wound healing.

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Human Keratinocytes Produce and Secrete IL-18

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Interleukin 18 (also known as IGIF (Interferon Gamma Inducing Factor) or IL-1γ) is a recently discovered cytokine which can enhance IFNγ production by natural killer- and T-cells in concert with IL-12. Because IFNγ plays an important role in many inflammatory skin diseases it is important to elucidate the role of mediators which regulate the production of this cytokine. Little is known about IL-18 regulation in human skin. IL-18 production by human keratinocytes was determined by using a human keratinocyte cell fine (HaCaT) Here we show that these keratinocytes constitutively produce intracellular proIL-18. The non-processed form of IL-18 was also detectable in the supernatant already after 3 h of culturing. Furthermore we showed that proIL-18 is also constitutively produced in high amount by normal human epidermal cells and that keratinocytes are the major source. Histochemical staining of IL-18 in normal skin shows diffuse staining in the epidermis, which is in line with the *in vitro* data. Western blotting and immunodetection showed that, compared to other tissue Eke normal human leukocytes and PBMC, Langerhans cells depleted epidermal cells produce much more proIL-18. These experiments show for the first time that human keratinocytes relative to PBMC or Leukocytes produce a considerable amount of proIL-18, which is also readably released. High constitutive levels of IL-18 might contribute to the predominantly Th 1 -like environment in human skin.

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Long-Term Culture of an Immature Functional CD4+ CD8+ IL7– Dependent Tumor T-cell Clone from a Patient with a Cutaneous T-cell Lymphoma

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Only few studies have reported the continuous long-term growth of human tumor T cell lines from patients with a cutaneous T cell lymphoma (CTCL). After informed consent and approval of an ethical committee, we cultured peripheral blood lymphocytes of a 50-y-old woman with an erythrodermic CTCL. Fifty percentage of uncultured blood lymphocytes had a CD3+, CD4+, CD8+, TCR Vβ22+ phenotype. An identical T cell clone was found in the skin and in the blood, using PCR-DGGE. After 2 mo culture with IL2/IL7, the T cell line contained 100% clonal tumor T lymphocytes. The phenotype of tumor cells, as determined by two- and three-color flow cytometric analysis, was CD2+, CD3+, CD4+, CD8αα+, CD28+, TCR Vβ22+, MHC class I+, MHC class II–. Tumor cells expressed low levels of IL-2 receptor α and β chain, but a high level of IL-2 receptor γ chain. They were Fas + but did not express CD94 and CD158a/CD158b. The growth of these lymphoma T cells was critically dependent of the presence of IL-7. High proliferation rates were observed with IL-7 and IL-4, whereas a lesser degree of proliferation was noted in presence of IL-2, and no response to IL-13. The tumor cells had a functional TCR, since they proliferated in response to coated anti-CD3 mAb. In addition, coated anti-CD3 mAb increased the T-cell response to IL-2. In conclusion, we have isolated a double positive, IL7-dependent, tumor T cell clone with a functional TCR/CD3. This long-term tumor T cell clone is very similar to normal immature functional T lymphocytes.

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Interaction of Dermal Fibroblasts and Eosinophils: Biologic Relevance of CC Chemokine Receptor 3 Ligands

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Increased numbers of eosinophils and dermal fibroblasts are found in different autoimmune diseases, particularly in sclerotic disorders of the skin. The reason for this phenomenon is still unclear, however, it is well know that fibroblasts are a major source of CC chemokines responsible for the attraction of eosinophils at the site of inflammation. On the hand, eosinophils are capable to release cytokines activating dermal fibroblasts leading to an amplification of this immune response. In this study we investigated the interaction of dermal fibroblasts and eosinophils with respect of the biologic activity of CC chemokines. RT-PCR revealed that cytokine-stimulated dermal fibroblasts are able to synthesize mRNA for RANTES, eotaxin and MCP-4, whereas mRNA for eotaxin-2 could not be detected. To investigate the biologic potency of these CC chemokines, actin polymerization, chemotaxis and the release of reactive oxygen species were assessed. In all experiments eotaxin-2 was as potent as eotaxin. Moreover, eotaxin-2-induced release of reactive oxygen species and intracellular calcium transients could be blocked by a monoclonal antibody against the CCR3 in the same range than eotaxin-stimulated eosinophils. This study demonstrates that there exists a discrepancy between the natural source of CC chemokines and their biologic role in the activation of human eosinophils: eotaxin-2 is one of the most potent CC chemokine for eosinophils, however, it could not be detected *in vivo*. Therefore, the most relevant CC chemokines for the interaction between dermal fibroblasts and eosinophils, so far, are eotaxin and RANTES.

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Human Keratinocytes Constitutively Express Interleukin-18 *In Vitro* and *In Vivo*

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Interleukin-18 (IL-18) is a potentially important immunomodulatory cytokine which shares many structural features with IL-1β and functions synergistically with IL-12 to induce IFNγ production from natural killer cells and T lymphocytes. It requires proteolytic cleavage by caspase 1 to attain biological activity. The IL-18 receptor is composed of a ligand-binding chain, IL-1 receptor related protein (IL-1Rrp) and a nonligand binding chain, termed Accessory Protein-Like (AcPL). IL-18 is secreted primarily by macrophages, but has also been reported in murine keratinocytes and Langerhans cells. However, there has been no data regarding human keratinocyte production to date. The aim of the present study was to define the production of IL-18 and its receptor by human keratinocytes.

Expression of IL-18 mRNA was determined by semiquantitative RT-PCR on second passage primary human keratinocytes derived from neonatal foreskins. IL-18 mRNA was constitutively transcribed and was not significantly altered following exposure to IL-1α, TNFα, IFNγ, PMA or nickel sulphate. By Western blotting, IL-18 was constitutively present in keratinocyte lysates and was detectable in supernatants in the unprocessed 24 kDa form. A human IL-18 ELISA demonstrated supernatant IL-18 levels between 100 and 800 pg per ml (n = 4). *In vivo* expression of IL-18 was assessed by immunohistochemistry utilising a panel of antibodies on neonatal foreskin and adult human skin. Diffuse staining was observed in all layers of epidermis, most marked in basal and granular cells. Primary keratinocytes expressed IL-1Rrp and AcPL constitutively at the mRNA level, although did not respond to treatment with IL-18, as assessed by failure to induce nuclear translocation of NFκB by band shift.

These results demonstrate that IL-18 is constitutively synthesised by human keratinocytes, both *in vitro* and *in vivo* and is secreted in an unprocessed form *in vitro*. The failure of keratinocytes to process IL-18 raises the question of the role of caspase-1 in keratinocytes. Release of IL-18 may permit them to regulate IFNγ production, the one immunomodulatory molecule which they appear unable to produce, during cutaneous inflammatory responses.

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Interleukin-1 (IL-1) Homeostasis in Murine Epidermis: Evidence from Transgenic Mice

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IL-1 is a potent proinflammatory cytokine constitutively expressed by keratinocytes. The IL-1 gene family consists of two proinflammatory ligands, IL-1α and IL-1β and a specific antagonist molecule, IL-1 receptor antagonist (IL-1ra). Although homeostatic regulation of this system has been suggested, such regulation has not to date been experimentally confirmed. To explore this issue, we have employed transgenic mice in which expression of IL-1α, IL-1ra and IL-1 receptor type-1 (IL-1R1) has been targeted to keratinocytes by the human keratin-14 promoter. In each line we have studied expression of IL-1α and IL-1ra by ELISA in total epidermal protein extracts. To examine release of each cytokine by intact epidermis, dispase-separated epidermal sheets were cultured overnight at 37°C and supernatants harvested for analysis.

Epidermal sheets from control and IL-1R1 transgenic mice secreted low levels of IL-1ra (358 $\pm$ 11 pg per ml and 436 $\pm$ 12 pg per ml, respectively) whilst increased levels were found in supernatants from IL-1ra transgenic epidermal sheets (6999 $\pm$ 260 pg per ml and 1581 $\pm$ 229 pg per ml in two separate lines). Epidermis from IL-1α transgenic mice also secreted increased IL-1ra (2058 $\pm$ 23.31 pg per ml). Expression of intracellular IL-1ra was found at low level in control, IL-1α and IL-1R1 mice (22 $\pm$ 0.5, 20 $\pm$ 0.25 and 18 $\pm$ 0.15 ng per ug total protein, respectively), with increased levels in the two IL-1ra transgenic lines (93 $\pm$ 2.5 ng per ug and 63 $\pm$ 0.6 ng per ug). Low levels of IL-1α (11.6 $\pm$ 0.34 pg per ml) were present in organ culture supernatants of control animals, with high level release in IL-1α transgenic mice (>500 pg per ml). Interestingly however, increased levels of IL-1α were secreted by epidermal sheets from both IL-1ra transgenic lines (42 $\pm$ 3 pg per ml and 35 $\pm$ 0.39 pg per ml).

These findings suggest a complex homeostatic mechanism in which not only increased levels of agonist lead to increases in antagonist production, but also that increases in antagonist release lead to increased production of agonist, underscoring the complexity of cytokine networks in epidermis.

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Reactivities of Contact Allergenic Quinones towards a Model Peptide and Identification of the Adducts

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Allergic contact dermatitis is a T-cell mediated immune response resulting from skin contact with low molecular weight organic or inorganic compounds. These low molecular compounds (haptens) have to be coupled to carrier proteins before they can activate the immune system. Although several thousands of haptens have been identified and the immune response of these are intensively studied the activation of the hapten and the reaction between the activated hapten and the protein are only partly investigated.

In this study we have used a hexapeptide (H-Pro-His-Cys-Lys-Arg-Met-OH) as a model of the carrier protein. The peptide includes all nucleophilic amino acid residues prone to react with activated electrophilic haptens. Reactive quinones were used as haptens. The formed adducts were isolated by HPLC and identified by NMR and MS.

The addition of the haptens was exclusively on the cysteine residue. The phenol-peptide adducts formed were to some part oxidized by excess of quinone to the corresponding quinone-adduct. Equal accessibility of all nucleophilic groups on the model peptide was shown by NMR.

Addition of low-molecular compounds to proteins has previously been shown to occur to the amino group of lysine. However, from a chemical point of view, the cysteine sulphydryl group is more reactive. The peptide used in this study permit a comparison of all nucleophilic amino acid residues. The results support cysteine as an important site of hapten conjugation on carrier peptides.

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Keratinocyte Nerve Growth Factor is Upregulated by Neuropeptides and During Cutaneous Wound Healing *In Vivo*

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Nerve growth factor (NGF) is an essential neurotrophic factor required for the growth and maintenance of cutaneous sensory nerves. Although it has been reported that keratinocytes are capable of producing NGF, little is currently known regarding regulation of NGF in the skin. We have recently shown that the neuropeptides neurokinin A (NKA) and substance P (SP) upregulate NGF mRNA and protein levels in murine PAM 212 keratinocytes. These neuropeptides are known to be released in the skin during inflammation and wound healing. Here we test the hypothesis that the release of neuropeptides *in vivo* and the experimental induction of wounds modulate the production of keratinocyte NGF in mouse skin. To test this possibility we topically administered capsaicin, which is known to induce the release of the neuropeptides NKA and SP from sensory nerves, on mouse (C57BL/6) skin and investigated the *in vivo* NGF expression by immunohistochemistry. Capsaicin treated mouse skin shows an upregulation of NGF expression compared to untreated skin. In order to determine the effect of wounding on keratinocyte NGF production, experimental wounds were generated then biopsied over time. The biopsied specimens were then established as explant cultures and ELISA studies were conducted in order to measure NGF protein levels in the supernatants. Supernatants of explant cultures demonstrate a peak of NGF protein production 2 h after wounding. In addition, *in situ* RT-PCR was performed on wound biopsies to determine NGF mRNA expression in regenerating epidermis. In situ RT-PCR demonstrated NGF mRNA expression in individual keratinocytes predominantly at the wound edges. Our findings further stress the importance of cutaneous neuropeptides in the regulation of keratinocyte NGF expression and suggest interesting implications for maintenance and regeneration of cutaneous nerves in normal skin, during inflammation and wound healing.

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DC-CK1/PARC mRNA is Overexpressed in Psoriatic Lesions

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Psoriasis is a common inflammatory skin disorder with a characteristic infiltrate of neutrophils, macrophages and activated T-lymphocytes in the dermis and epidermis. T-lymphocyte activation and lymphokine production are supposed to be crucial in the persistence of epidermal hyperplasia and inflammation. Lymphocytes extravasate the bloodstream and undergo a multistep process regulated by different adhesion molecules on endothelium cells and T-cells as well as by several chemokines. The selective T-lymphocyte chemoattractant chemokine dendritic cell-chemokine1/pulmonary and activation-regulated chemokine (DC-CK1/PARC) is known to be expressed in alveolar macrophages, follicular dendritic cells of germinal centers and stimulated peripheral blood monocytes. In this study we examined the amounts and sources of DC-CK1/PARC in psoriatic plaques, nonlesional skin and normal human skin by RT-PCR, Northern blot analysis and *in situ* hybridization (ISH). We detected DC-CK1/PARC mRNA overexpression in psoriatic lesions compared to both controls. We especially found increased expression in macrophages surrounded by mainly CD3+, CD4+, CD45RO+ memory T-cells, while epidermal Langerhans cells did not show DC-CK1/PARC mRNA expression. In nonlesional skin we could not find mRNA by ISH and only low levels as measured by RT-PCR. So, the recently described chemokine DC-CK1/PARC could be detected in macrophages of psoriatic plaques. This chemokine may play an important role in the recruitment of skin infiltrating T-lymphocytes through a positive perivascular chemokine gradient and lead to emigration of T-lymphocytes from the bloodstream into the skin and is possibly involved in the pathogenesis of this T-cell mediated inflammatory skin disease.

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T cell Infiltration in Evolving Psoriatic Plaques Induced by Interferon-gamma

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Considerable evidence suggests that interferon-gamma (IFN-γ) is a key cytokine in pathogenetic mechanisms in psoriasis. Since psoriasis is manifest only in certain individuals, it might be expected that the skin of psoriatics would have an altered response to IFN-γ. To address this a scanning laser Doppler, which measures cutaneous blood flux, was used to identify the active, advancing edge of psoriatic plaques in eight patients with deteriorating psoriasis. Human recombinant IFN-γ and saline control were injected intradermally into clinically normal skin immediately in front of the advancing plaque edge, into skin distant from the plaque, and into anatomically matched sites in nine healthy volunteers. Biopsies were taken after 48 h and assessed cytochemically.

IFN-γ induced increased CD3 positive T cell infiltration at all injection sites, compared to saline controls. This pro-inflammatory effect was exaggerated in front of the advancing plaque edge compared to distant skin (mean cells/biopsy 477 *vs* 320; $p < 0.01$), but there was no difference between normal psoriatic skin and control skin. Tissue-specific T cell homing was assessed by determining expression of the skin homing receptor, cutaneous lymphocyte-associated antigen (CLA) and the integrin, $\alpha_E\beta7$, which is associated with the localisation of T cells at noncutaneous, mucosal sites. IFN-γ induced nonspecific T cell infiltration, which was maximal at the plaque edge (17.2% of cells CLA +ve, 35.3% $\alpha_E\beta7$ +ve) compared to equivalent saline injected skin (47% CLA +ve, 9.2% $\alpha_E\beta7$ +ve). Consistent with this, IFN-γ did not alter expression of the endothelial ligand for CLA, E-selectin, in any of the biopsies.

We have previously shown that CLA positive cells infiltrate early and specifically in evolving plaques, whereas established plaques contain a lower percentage of CLA positive lymphocytes. Because IFN-γ induced a nonspecific T cell infiltrate, this study suggests that IFN-γ does not play a primary pathogenic role in evolving psoriatic lesions, but may instead have secondary effects in more established plaques.

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Accumulation of Neutrophilic Polymorphonuclear Leukocytes in the Skin of Interleukin-8-Producing Mycosis Fungoides

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Epidermotropic cutaneous T cell lymphoma (CTCL) cells lines usually exhibit a Th2 differenciation. However, the Th2 functional pattern cannot account for unusual clinicopathological forms of CTCL, such as the pustular form which is characterized by the accumulation of neutrophils in tumoral lesions. We report two patients presenting with this latter form of CTCL. In both patients, tumoral T cells expressed CD4+ with loss of CD5 and CD7 antigens. 25–50% of cells infiltrating the skin lesions were neutrophils *versus* less than 10% in control patients with non pustular CTCL ($n = 6$). A dominant GV-GJ clonal rearrangement of the TCRG locus was detected in the skin lesions of both patients by using PCR, whereas the same rearranged DNA segment was also detected in the blood of one patient. A T cell line enriched for tumoral cells derived *in vitro* from the peripheral blood of this latter patient (AK1) was shown to include the same monoclonal rearrangement. In situ immunohistochemical analysis of skin lesions showed a strong cytoplasmic staining of the dermal infiltrate with an anti-IL-8 monoclonal antibody in both patients, while no immunoreactivity was found in control CTCL cases ($n = 6$). RT-PCR analysis of the AK1 cell line allowed the detection of IL-8 transcripts, while this search was negative among three control CTCL lines derived from cases showing no neutrophilic skin infiltrate. Immunocytochemical analysis with anti-IL-8 mAb revealed cytoplasmic staining of AK1 cell line while no staining for IL-8 was found in three control Sezary lines. By immunochemistry analysis, no significant difference was found in the skin, in AK1 and in control Sezary lines for expression of RANTES and GM-CSF between the patients and controls.

Altogether, these data suggest that the recruitment of neutrophils in skin lesions of pustular mycosis fungoides are related to the production of interleukin-8 by malignant cells.

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Early Signals in the Nickel Activation of Peripheral Blood Mononuclear cells in Nickel-Allergic Subjects

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The lymphocyte transformation test has been used in an attempt to diagnose nickel allergy *in vitro*. However, the greatest problem hitherto has been the unspecific stimulation of lymphocytes from control subjects without clinical symptoms of nickel allergy. In has previously been reported about different cytokine profiles in antigen-stimulated lymphocytes. The aim of the present study was to correlate levels of DNA synthesis and cytokines, IL-2, IL-4, TNF-beta and IFN-gamma, in short time (up to 72 h) cultured nickel-stimulated peripheral blood mononuclear cells from seven nickel-allergic patients, with a positive patch test to nickel sulphate with erythema, oedema, papules and vesicles, and seven control subjects. The patch test had been performed, in both patients and control subjects, within 1 y in relation to the *in vitro* study. The cells were purified from peripheral venous blood by density gradient centrifugation on Ficoll-Paque and cultured for 5, 10, 24, 48 and 72 h, in the presence of nickel sulphate or saline. The proliferative rate was determined as the uptake of tritiated thymidine into DNA. The production of cytokines in the supernatants of the cultures was measured by ELISA. There was a statistically significant ($p < 0.01$) difference in the DNA synthesis between the patients and control subjects at 72 h, and at the same time also a difference in the concentrations of TNF-beta ($p < 0.05$) and IL-4 ($p < 0.01$). Already at 24 h and proceeding through the remaining culture period, there was a statistically significant ($p < 0.001$) difference in the concentrations of IL-2. Thus, IL-2 may be regarded as a critical and early occurring cytokine when trying to diagnose nickel allergy *in vitro*.

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Phase I/II Trial in Disseminated Melanoma with Anti-GD2 Monoclonal Antibody – Monotherapy, in Combination with GM-CSF and Interleukin-2 Fusion Protein

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The rationale for the clinical use of the anti-GD2 (ch14.18) monoclonal antibodies in melanoma is based on the findings that this reagent can mediate complement-dependent cytoxicity (CDC) and antibody-dependent cytoxicity (ADCC) of effector cells against tumour cell targets. In a phase I/II trial we treated six patients with ch14.18 and six patients with a combination of ch14.18 plus rhGM-CSF. The 12 patients with metastatic malignant melanoma received ch14.18 intraveneously over 6 h per day over 10 d. Six of the 12 patients received additionally subcutaneous injections of rhGM-CSF for 10 days. This treatment regimen was repeated three times after a therapy-free period of 3 wk. Side-effects were related to ch14.18 infusion and consisted of pain, hypertension and headache at a dose of 40 mg per m^2. No augmentation of side-effects were seen in patients receiving ch14.18 plus rhGM-CSF up to a single dose of 400 mcg per m^2. Increase in white cell count occurred after rhGM-CSF. No HAMAs could be detected. No significant antitumour activity was seen. In all patients treated with ch14.18 plus rhGM-CSF, even a considerable tumour progression was observed. We conclude that the treatment with ch14.18 given intraveneously as monotherapy, or in combination with GM-CSF is not effective in heavily pretreated patients with multiple metastases. A phase-I dose-escalation study with the immunocytokine fusion protein ch14.18-IL2 has been started.

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Dithranol Upregulates IL-10 Receptors on Human Keratinocytes

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Dithranol is highly effective in the treatment of psoriasis, however, its mode of action is still not well known. Since IL-8 and IL-10 are involved in the pathogenesis of psoriasis, the aim of our study was to investigate the effect of dithranol on IL-8/IL-10 production and IL-10 receptor expression of keratinocytes. Cultured HaCaT cells were treated with 0.1–1 μg per ml dithranol for 30 min. After 1 and 3 h total cellular RNA isolated from HaCaT cells was reverse transcribed (RT) to cDNA which was subjected to polymerase chain reaction (PCR) with specific primer pairs for IL-8/IL-10 and IL-10 receptor. For immunohistochemistry cultured HaCaT cells were stained with a monoclonal antibody against human IL-10 receptor. Our results showed that dithranol treatment did not change the highly elevated level of IL-8 mRNA of HaCaT cells and IL-10 mRNA signal with RT-PCR could not be detected. Depending on the concentration, dithranol increased the mRNA production of IL-10 receptors in HaCaT cells. This dithranol induced IL-10 receptor upregulation was also observed on the protein level in a dose dependent way using immunohistochemistry. Since the IL-10 receptor expression of keratinocytes in psoriatic lesional skin is downregulated, the dithranol induced upregulation of the receptor might be involved in the therapeutic action of the drug.

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Effects of Neuropeptides on IL-8/IL-8 Receptor System in Cultured Human Keratinocyte Cell Line HaCaT and Dermal Fibroblasts

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The neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP) and α-melanocyte-stimulating hormone (α-MSH) are known to be able to regulate the production of cytokines in the skin. Since IL-8 plays an important role in cutaneous inflammation, the effects of SP, CGRP and α-MSH on the IL-8/IL-8 receptor (CXCR1) systems of these cell types were studied. Cultures of human dermal fibroblasts and a keratinocyte cell line HaCaT were treated with 10^{-8} M SP, CGRP or α-MSH. The results demonstrated that these neuropeptides have different effects on the IL-8 and CXCR1 expressions of these cells. SP and CGRP upregulated the CXCR1 mRNA expression in HaCaT cells, but had no influence on their IL-8 production, whereas α-MSH had no effect on either the IL-8 or the CXCR1 mRNA expression in HaCaT cells. In contrast α-MSH resulted in a time-dependent induction of the IL-8 mRNA expression in dermal fibroblasts. This induction was already detectable after 3 h, and after 6 h there was a 5-fold change in comparison with the controls. The IL-8 content of the supernatant was also in-creased, with a maximum at 48 h after α-MSH treatment. The data established in the present study support the notion that neuropeptides can directly modulate the IL-8/IL-8 receptor system of keratinocytes and fibroblasts.

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Immunomodulatory Activity of *Avena Rhealba*: Interest in Skin Inflammatory Disorders

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The immune mechanisms which play a central role in the pathogenesis of atopic dermatitis and other inflammatory dermatoses, are centered around the activation of T lymphocytes and result from the complex interaction of several cells including keratinocytes, endothelial cells, eosinophils, Langerhans cells and T cells, as well as numerous cytokines and mediators. The both role of T-helper 1 (Th1) and Th2 cells which differ in their secretory patterns of cytokines is postulated in the disease process, albeit at different stages, and make the use of topical potent immunosuppressive agents logical therapeutic considerations.

The aim of this work was to evaluate the activity of *Avena Rhealba* extracts on immune cellular responses. In the present study, two models have been investigated: (1) the expression by cultured human keratinocytes cell line of the anti-inflammatory cytokine transforming growth factor β (TGFβ1) which is a potent regulator of lymphocyte regulation, plays a negative regulatory role on the development of Th2 cells and is able to induce an immune privilege (2) the production by human peripheral blood mononuclear cells (PBMC) of interleukin 2 (Th1 cytokine) and interleukin 4 (Th2 cytokine) which is up-regulated in atopic dermatitis.

For each model, *Avena Rhealba* extracts were prepared in culture medium and evaluated at different concentrations. *Avena Rhealba* extracts significantly induced TGFβ1 production (+30% to +70% for 24 h exposure and according to the extract) by keratinocytes; *Avena Rhealba* was also able to significantly down-regulate IL2 and IL4 production (–20% to –80% for 18 h exposure and according to the extract) by PBMC. Our results demonstrate that *Avena Rhealba* extracts are potent regulator of immune responses and suggest that *Avena Rhealba* topical preparations could be able to improve atopic dermatitis disease.

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Eotaxin in Bullous Pemphigoid of Three Month Duration with Tissue Eosinophilia

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Bullous pemphigoid (BP) is an autoimmune blistering disease characterised by large tense blisters on erythematous macules or urticarial plaques and circulating IgG autoantibodies reacting with hemidesmosomal proteins of the basement membrane zone (BMZ). In contrast to pemphigus vulgaris, eosinophils make up a major component of the cellular infiltrate. Eotaxin 1 and 2 are recently discovered polypeptides which upon binding to the CCR3-receptor induces both, activation and chemotaxis of eosinophils as well as IL-4 and IL-5 secretion by TH2-lymphocytes *in vitro*. Its pathological role in tissue eosinophilia has not been studied so far.

We studied a 54-y-old patient suffering from BP with generalised blister formation of three months duration sparing the mucous membranes. The clinical diagnose was confirmed by demonstration of tissue band and circulating anti-BMZ antibodies of the IgG class. Histological examination revealed vacuolic degeneration of the basal layer with blister formation and a dermal lymphohistiocytic infiltrate with numerous eosinophils. No eosinophilia was observed in peripheral blood, but microscopic inspection of the blister fluid revealed numerous eosinophils.

To test for a role of eotaxin 1 in the induction of tissue eosinophilia we compared eotaxin level in serum and blister fluid by ELISA. Serum eotaxin 1 level was below detection, while eotaxin 1 level in the blister fluid was markedly elevated at 372 pg per ml. The mast cell products ECP and tryptase were increased 12-fold and 8-fold in the blister fluid *versus* serum. CCR3-expression on eosinophils in tissue has been quantified.

We demonstrate here that eotaxin 1 is locally increased in BP and might play a role in trafficking of eosinophils in tissue eosinophilia.

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Secretion of Aminoterminal Truncated Eosinophilic Chemokines and Gene Expression of the Ectopeptidase Dipeptidyl Peptidase IV (CD26) by Dermal Fibroblasts Indicate CD26 Involvement in Skin Inflammation

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We recently discovered, that dermal fibroblasts are a cellular source of RANTES and eotaxin and demonstrated their transcriptionally regulation upon stimulation. Both eosinophilic chemokines potentially play a role in inflammatory skin diseases characterized by selective eosinophil infiltration into the dermis. Significant amounts of RANTES and eotaxin are found in aminoterminal truncated form. We asked, whether the ectopeptidase CD26, which has been shown to truncate these chemokines *in vitro*, does play a role in human skin and skin diseases. CD26 mRNA expression in dermal fibroblasts and keratinocytes was assayed using semiquantitative RT-PCR.

We found temporarily increased constitutive CD26 mRNA expression in dermal fibroblasts after short-term (6 h) stimulation with TNFα, IL-1α, IFNγ or IL-4. Fewer number of RT-PCR cycles were necessary to amplify fibroblast derived CD26 mRNA compared to CD26 mRNA derived from cultured normal human skin keratinocytes (NHK) indicating significant stronger CD26 gene expression in dermal fibroblasts. No CD26 mRNA expression was detectable in the human keratinocyte cell line HaCaT. Since truncated eotaxin and RANTES display reduced eosinophil chemotactic activity and in the case of RANTES changed receptor specificity, these results suggest, that fibroblast derived CD26 activity does play a regulatory role in eosinophil upregulated inflammation by changing the RANTES/eotaxin activity profile in skin.

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Chemokines are Differentially Expressed by Human Dermal Fibroblasts

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In human skin, both resident and migratory cells are centrally involved in dermal inflammatory processes by participating in intricate cytokine networks. Fibroblasts release diverse peptide mediators including different chemokines and thus are able to focus and amplify local inflammatory responses. In this study, the involvement of fibroblasts was further evaluated by monitoring the differential induction of C-C chemokine RANTES and C-X-C chemokines IL-8 and IP-10. Respective mRNAs and peptides were detected by RT-PCR and sensitive and specific ELISAs. IL-8 peptide was shown to be expressed as early as 4–6 h after stimulation with either TNFα or IL-1β, whereas IP-10 and RANTES appeared much later (after 48 and 72 h, respectively). At this time interval, peptide levels differed at log scale (IL-8: RANTES:IP-10 at 100:10:1). Interestingly, among 30 different fibroblast donors, 10 percentage failed to express RANTES even after long-term and high dose stimulation with IL-1β. In contrast, IFN α and γ were able to induce IP-10 only. TH-2 cytokines IL-4 and 5 or IL-10 and 12 remained without stimulatory effects on any chemokine. These data demonstrate that human dermal fibroblasts consist of functionally in homogenous cell populations which are able to differentially respond to inflammatory stimuli.

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Intracytoplasmic Interleukin-10 in Perpheral Blood T-Lymphocytes of Mycosis Fungoides Patients

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Semiquantitative RT-PCR analysis revealed a stage-dependent increase in IL-10 mRNA in lesional skin of mycosis fungoides and local IL-10 showed higher amounts in mycosis fungoides than in other nonmalignant skin infiltrates. As the contribution of IL-10 to the pathogenesis of mycosis fungoides is still a matter of discussion we analysed the quantity intracytoplasmic IL-10 in peripheral blood T-lymphocytes of 13 patients with advanced stage (plague/tumor) mycosis fungoides and compared these results with 10 healthy control persons. We used flow cytometry with intracytoplasmic IL-10 staining for CD3, CD4, CD8, and CD45RO positive T-cells. Semiquantitative RT-PCR was performed to analyse IL-10 mRNA in lesional skin of five mycosis fungoides patients and in normal skin. As reported before the IL-10 mRNA levels were higher in lymphoma infiltrates than in normal skin in all cases. There were no significant differences of peripheral blood lymphocytes for intracytoplasmic IL-10 regarding CD3+ cells (average number of CD3/IL-10-positive cells: 4.0% pat./4.6% controls), CD4+ cells (5.4% pat./4.9% controls) and CD8+ cells (4.8% patients/7.7% controls) between mycosis fungoides patients and controls. Regarding the CD45RO-positive cells we found an average of 22.4% of cells positive for IL-10 in the mycosis fungoides patients group compared with 11.4% in the control group. Immunohistochemical analysis revealed large numbers of CD45RO-positive T-cells in all skin infiltrates of mycosis fungoides analysed. As increasing IL-10 levels mRNA were reported to be associated with lymphoma progression the CD45RO+ cells might provide a microenvironment leading to downregulation of immunologic tumor surveillance in mycosis fungoides infiltrates by releasing this immunosuppressive cytokine. Single cell analysis of these CD4RO+ cells has to prove this proposal.

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In Situ RT-PCR Detection of Human Cytokine mRNA in Cutaneous Graft-*Versus*-Host Disease

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In situ PCR techniques allow the detection of specifically amplified nucleic acid sequences in individual cells within a heterogenous cell population. The ability to localize target sequences and correlate them spatially with tissue and cell morphology is highly useful in the study of cell-specific gene expression and opens new perspectives in studying cytokine-mediated pathomechanisms. Graft-*versus*-Host disease (GvHD) after allogenic bone marrow transplantation is considered as paradigm of a cytokine-induced and maintained disease. Therefore, we performed a reverse transcription technique with subsequent PCR amplification for distinct cytokines, i.e., TNF-α, IFN-γ, IL-10, TGF-β and macrophage-derived chemokine (MDC) in frozen sections from 18 patients with acute or chronic, lichen-planus(LP)-like cutaneous (c)GvHD. In sections from LP-like GvHD MDC, IFN-γ and TGF-β expressing cells were observed in considerable numbers in the upper dermis and perivascularly whereas in acute cGvHD significantly less signals were detected. The amount of *in situ* expression of IL-10 was also high in LP-like cGvHD and, in addition, increased from stages II to IV of acute cGvHD. No significant differences were observed with regard to TNF-α mRNA expression in acute and LP-like cGvHD. The higher level of expression – at least on a semiquantitative level – may reflect the higher numbers of lesional producer cells. However, the increase in *in situ* expression of IL-10 with increasing severity of acute cGvHD may be explained by an upregulation of this cytokine at the transcriptional level rather than by a mere increase of IL-10 producing cells. As altered cytokine secretion patterns apparently contribute to the main pathologic features of cGvHD, e.g., apoptosis, acanthosis and/or fibrosis, the *in situ* PCR techniques will be helpful in more detailed investigations.

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Infiltrating TH-1 Cells in Psoriasis Express the Chemokine Receptor CXCR3

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Chemokines and chemokine receptors are known to play a central role in recruitment and activation of leukocytes during inflammation. Recently chemokine receptors have been shown to be differentially expressed on naive T cells and polarized T cell subsets. Psoriasis is a chronic inflammation disease where infiltrating T cells of the TH-1 type are believed to be of importance for the induction and maintenance of the psoriatic phenotype.

We investigated tissue samples of psoriatic skin for the *in situ* expression of various CXC and CC chemokine receptors and corresponding ligands by immunohistochemistry.

Immunohistochernical staining demonstrated expression of CXCR3 on the majority of infiltrating T cells, while CCR3 and CCR5 expression was not detectable. Additionally we found an of expression the CXCR3 chemokines MIG and IP-10 in dermis of lesional skin. Flowcytometry analysis of cell suspensions obtained from lesional skin showed a co-expression of CD3 and CXCR3. Furthermore we were able to detect the TH-1 cytokine IFN-γ but not the TH-2 cytokine IL-4 in CXCR3 positive T cells by intracellular flowcytometry staining. Interestingly in a patient treated with the Vitamin D analogue Calcipotriol, infiltrating T cells produced high levels of IL-4 but not IFN-7.

We propose that one mechanism involved in the pathophysiology of psoriasis may be chemoattraction of CXCR3 bearing T cells into lesional skin. Detection of IFN-γ in CXCR3 positive infiltrating T cells establishes a link between disease specific T cell migration and their functional differentiation state as TH-1 cells.

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Interleukin One Alpha Stimulates Transglutaminase Activity in Cultured Human Outer Root Sheath Keratinocytes and in the Isolated Human Sebaceous Infundibulum

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In view of the effects of IL-1α on the sebaceous infundibulum we examined its effects on transglutaminase activity and expression in isolated human outer root sheath (ORS) keratinocytesand sebaceous infundibula.

The addition of 2 mM $CaCl_2$ to ORS keratinocytes promoted a rise in transglutaminase activity that peaked at 48 h and fell back to control levels. The addition of IL-1α caused a dose-dependent rise in transglutaminase activity which again peaked at 48 h. Lactate dehydrogenase (LDH) activity was unaltered by the additions, indicating a specific effect on transglutaminase activity. Control levels of transglutaminase activity rose in isolated human sebaceous infundibula after 48 h of maintenance and this rise could be inhibited, in 50% of subjects, by 1 μg per ml interleukin-1 receptor antagonist protein. The addition of IL-1α caused a dose-dependent increase in transglutaminase activity in isolated infundibula after 24 h which fell back to control levels over 96 h. IL-1α did not affect the pattern of expression of transglutaminase mRNA or protein. LDH activity was unaffected by IL-1α.

IL-1α may therefore have a role in normal and abnormal keratinocyte cornification.

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UVA-Mediated Lipid and Protein Oxidation in Human Blood after Extracorporeal Photoimmunotherapy

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Extracorporeal photoimmunotherapy (ECPI) is proven to be a new and efficacious alternative in the treatment of cutaneous T-cell lymphomas, scleroderma, GvHD, and others. The underlying mechanism is unclear, but the involvement of reactive oxygen species (ROS) is considered to be crucial in photopheresis. The present work was performed to find out PUVA specific changes in unspecific (MDA) and specific (monohydroxyeicosatetraenoic acids, HETEs) lipid peroxidation and oxidative modification of proteins in plasma samples.

In vivo experiments included 10 patients with cutaneous T-cell lymphoma and progressive systemic scleroderma. For *in vitro* conditions buffy coat and plasma from patients were diluted according to photopheresis conditions and exposed to increasing doses of UVA and different 8-MOP concentrations. Carbonyls were measured using electrophoretic methods and HETEs by NICI-GC-MS.

Exposure of buffy coat and plasma to the selected UVA doses in combination with various 8-MOP concentrations resulted neither in an increase of MDA as marker of lipid peroxidation nor in enhanced DNPH-reactive protein carbonyls as markers of oxidatively modified proteins. Interestingly, the effect of ECPI-treatment on the formation of specific lipid peroxidation products reveals the increase of HETE isomers. The following monohydroxy metabolites were generally identified in plasma samples: 2-, 3-, 5-, 8–12-and 15-HETE. Under *in vitro* conditions it could be established that HETE-formation increased in a dose dependent manner with increasing UVA-doses and 8-MOP-concentrations as well.

The present results suggest that, although ROS are formed during ECPI, gross oxidative damage of lipids (MDA) and proteins (carbonyls) does not occur. However, specific oxidation products of arachidonic acid were highly increased and may cotrigger the photoimmunomodulary effects of ECPI.

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Interleukin-15 Expression in Cutaneous T-Cell Lymphoma

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Interleukin-15 (IL-15) is a 14–15 kDa cytokine of the four-alpha-helix bundle family which shares many biological activities with IL–2: both support proliferation of T cells and enhance the cytolytic functions of CD8+ T cells and natural killer cells. Unlike IL-2 however, IL-15 mRNA have been identified in a variety of tissues and cell lines including epithelial cells. This ubiquitous expression of IL-15 mRNA doesn't correlate with a widespread expression of IL-15 protein; indeed, IL-15 expression is regulated at multiple levels.

In a previous study, we have observed that IL-15 protein was expressed in cutaneous T-cell lymphoma (CTCL). The aim of this work was to determine whether a correlation exists between the level of IL-15 protein expression and stage of the CTCL or degree of disease's remission under alpha-interferon therapy.

We examine nine parapsoriasis (PP) and 36 CTCL (13 Mycosis fungoides (MF) stage Ib, 12 MF stage IIb and 11 Sézary syndrome (SS)) for IL-15 protein expression by immunohistochemistry. Nineteen patients (eight MF Ib, six MF IIb and five SS) were under alpha-interferon therapy. We detected IL-15 protein expression at a moderate level for the majority of the patients (eight PP, 11 MF Ib, 10 MF IIb and nine SS). Only one patient with a parapsoriasis and six patients with CTCL (two MF Ib, two MF IIb and two SS) expressed higher levels of IL-15 protein. This various expression of IL-15 protein doesn't seem to correlate with the stage of the disease or with the inflammatory state or the density of the dermis inflammatory infiltrates. After alpha-interferon therapy, most patients (13 of 19) expressed higher levels of IL-15 protein. We still have to determine why some patients with CTCL express higher levels of IL-15 protein than others.

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Group A Streptococcal Reactive Dermal CD4+ T-Cells in Skin Lesions of Chronic Plaque Psoriasis Produce IFN-γ

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Recently, we have demonstrated that group A streptococcal antigen reactive T-cells are present in skin lesions of chronic plaque psoriasis, in common with those of the acute guttate form of the disease. To determine the cytokine profile (IFN-γ, IL-4 and IL-10) of these T cells in response to streptococcal antigen, T cell lines (TCL) were cultured from untreated lesional skin of 10 patients with chronic plaque psoriasis and four patients with other inflammatory skin diseases. TCLs were incubated with or without a sonicated, heat-killed mixture of group A streptococcal isolates (Strep-A) for 18 h in the presence of monensin, a transport inhibitor which prevents cytokine release, double stained for surface CD4 or CD8 and intracellular cytokine expression, and analysed by flow cytometry.

Psoriatic TCL were predominately $CD4^+$ (64–85%) with 10–32% $CD8^+$ T-cells. Variable numbers of $CD4^+$ T-cells produced IFN-γ (1.2–35%, median 13.9%) in nine of 10 TCL. Small numbers of IL-10-positive (0.8%–1.3%) and IL-4-positive (2.1–2.5%) $CD4^+$ T cells were also present in two of five and three of five psoriatic TCL, respectively. Cytokine-positive $CD8^+$ T-cells were rarely observed. In contrast, $CD4^+$ T cells in only one of the disease control TCL produced IFN-γ in response to Strep-A.

These findings demonstrate a TH_1 cytokine response to streptococcal antigens by $CD4^+$ T-cells in skin lesions of chronic plaque psoriasis.

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Interleukin-8 is Increased in Serum of Patients with Active Adamantiades-Behçet's Disease and is Released by Small Vessel Endothelial Cells

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Serum levels of several cytokines were assessed in 94 patients with Adamantiades-Behçet's disease (ABD), aged 11–68 y, in the active ($n = 75$) and the nonactive ($n = 19$) phase of the disease. Seventy-five healthy individuals matched for gender and age served as controls. In addition, the role of dermal microvascular endothelial cells in interleukin-8 (IL-8) secretion was investigated. Immortalized human dermal microvascular endothelial cells (HMEC-1) were challenged *in vitro* with serum samples of 18 ABD patients, serum of three healthy controls, IL-1β, tumor necrosis factor-α (TNF-α) and IL-8. Cytokine levels in serum samples and culture supernatants were determined by commercially available ELISA kits. Significantly increased serum levels of IL-8 were determined in patients with active ABD, especially when oral ulcers ($n = 51$, median 34 pg per ml, CI 0–324 pg per ml; $p = 0.0001$) and/or neurological signs ($n = 4$, median 71 pg per ml, CI 30–112 pg per ml, $p = 0.01$) were present, in comparison to patients in nonactive stage (median 0 pg per ml, CI 0–97 pg per ml) and to healthy controls (median 0 pg per ml, CI 0–45 pg per ml). No statistically significant differences could be obtained in serum levels of IL-1α, IL-1β, TNF-α, soluble ICAM–1, interferon-γ and basic fibroblast growth factor among patients with active *versus* nonactive disease and healthy controls. On the other hand, increased IL-8 secretion was found after incubation of HMEC-1 with serum from 16 of 18 patients tested (median 20 pg per ml, CI 0–61 pg per ml) compared to healthy controls (median 2.7 pg per ml, CI 2–3.4 pg per ml; $p < 0.05$). IL-1β, TNF-α and IL-8 failed to induce IL-8 secretion by HMEC-1. These data are suggestive of a notable correlation between IL-8 serum levels and the active oral and neurological manifestations of ABD patients and they are indicative of an apparent potency of ABD patients' serum to trigger small vessel endothelial cells to release increased amounts of IL-8.

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Dithranol Dose-Dependent Release of IL-1α from Keratinocytes as a Possible Mechanism Explaining the Drug's Well-Known Potential for Skin Irritation

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Dithranol (anthralin) is a classic, highly efficacious topical antipsoriatic, however, its application is hampered by its irritative potential, staining properties as well as chemical instability. The aim of the present study was to contribute to the further clarification of the mechanisms by which dithranol exerts its irritative effects. For this purpose hyperproliferative HaCaT keratinocytes were studied as an *in vitro* model for disturbed epidermopoiesis in psoriasis. The cells were incubated for 2 h with dithranol at various concentrations using acetone as a solvent (0.1% final concentration). The supernatants were harvested after additional 4, 24 and 48 h, and analyzed for the content of IL-1α by an ELISA technique.

As compared to the solvent-treated controls which were set to 100%, dithranol concentrations of 1, 3 and 10 μM led to a dose-dependent IL-1α increase of 145, 271 and 532% at 24 h as well as of 2641, 6347 and 8811% at 48 h (means of $n = 3$ independent experiments). After the first 4 h following the incubation there was no substantial difference of the IL-1α content in the supernatants for any dithranol concentration compared to the controls.

IL-1α is known as a major pro-inflammatory cytokine which is synthesized and stored in keratinocytes in high amounts. Our study demonstrates that the induction of the release of IL-1α from keratinocytes by dithranol may be regarded as an essential mechanism of the drug's potential to provoke unwanted skin irritation. This phenomenon was proven to be time-and dose-dependent which is in good correlation with the general clinico-therapeutic experience upon dithranol's application. Moreover the data confirm former *in vitro* observations that the drug leads to significant disturbances of the plasma membrane integrity allowing leakage of cytosolic components, e.g., lactate dehydrogenase (*Arch Dermatol Res* 282:325, 1990).

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The Elevated Levels of Soluble Interleukin-2 Receptor in Alopecia Areata are Not Correlated with the Type and Duration of the Disease

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The cell-mediated immunity has been found to play an important role in the pathogenesis of alopecia areata. Levels of soluble interleukin-2 receptor (sIL-2R) were measured as a sign of T-cell activation in sera of 50 (29 with patchy type of alopecia areata-AAV and 21 with alopecia areata totalis/universalis – AAT/U) patients with alopecia areata (AA) and in healthy control subjects. The patients were also divided into two groups with duration of disease less than 12 mo and above. The concentrations of sIL-2R were determined by ELISA method with sIL-2R kit. The t-Student and C-Cochran-Cox test were used to compare concentrations of sIL-2R in all groups. The results of the study showed mean concentrations of sIL-2R: 2011.6 pg per ml ± 1297.0 in AA patients, 2196.0 pg per ml ± 1582.8 in AAV, 1756.0 pg per ml ± 657.9 in AAT/U and 974.0 pg per ml ± 479.7 in the control group. The differences between all the AA groups and the controls were statistically significant with $p < 0.01$. There was no statistically significant difference in sIL-2R levels between patients with AAV and AAT/U. The mean concentrations of sIL-2R in patients with the duration of AA less than 12 mo and longer than 12 mo were 1978.3 pg per ml ± 1171.5 and 2076.3 pg per ml ± 1509.0, respectively. The differences between both groups and the controls were statistically significant ($p < 0.01$). The elevated levels of sIL-2R in AA patients suggest that lymphocyte activation is involved in pathogenesis of alopecia areata although we found no correlation between the sIL-2R levels and the type or duration of the disease.

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Interleukin 8 is Induced in Skin Equivalents Parallel to Differentiation

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The chemokine interleukin 8 (IL-8) is expressed by human keratinocytes both *in vitro* and *in vivo*. By use of specific monoclonal antibodies, immunohistochemical reactivity could be demonstrated in human epidermis which is modulated by degree of inflammatory reaction as well as keratinocyte differentiation. In this study, immunreactivity was monitored immunhistochemically in both normal human skin of different body locations as well as in dermo-epidermal raft cultures at different stratification and differentiation levels. No gross difference of chemokine expression was found among different locations irrespective of number of epidermal layers or levels of cornification. In raft cultures IL-8 was found not to be expressed at early culture intervals up to two to three days or 2–4 epidermal layers. With increasing culture duration, IL-8 became detectable in a pattern comparable to normal skin after 12–14 d. These data show that IL-8 undergoes differential expression in parallel to the keratinocyte differentiation levels. There appears to exist an IL-8 linkage to terminal differentiation products so far not characterized. Thus, IL-8 may participate in normal tissue turnover and growth regulation.

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Detection of Chemokine IP-10 in Serum of Patients with Different Skin Diseases

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The interferon γ inducible peptide 10 Kd (IP-10) has been known for a long time. However, its biological function and cell origin have only recently been studied to a greater extent. Data have shown that IP-10 activates T-lymphocytes and exerts angiogenic effects. Accordingly, it may be involved in inflammatory processes as well as tumor progression. So-far, circulating IP-10 has hardly been studied in humans. Using monoclonal antibodies, a specific and sensitive ELISA was established to measure IP-10 levels in diverse human biological fluids. Serum samples from patients with malignant melanoma (n = 100), psoriasis (n = 50), erysipelas and herpes zoster (n = 20) were examined. 70% of melanoma sera were found to contain IP-10 levels well above 0.3 ng per ml up to 6,7 ng per ml. In contrast, only 10% of psoriatic and 40% of sera from patients with erysipelas and zoster were found to contain elevated IP-10 levels. Apparently, circulating IP-10 is not involved in chronic inflammatory processes nor in acute infectious processes of the skin. In contrast, the results indicate that IP-10 may be involved in melanoma possibly due to effects on angiogenesis or tumor cells themselves. Further studies have however, to be performed to study both specificity as well as correlation to disease activity for this tumor.

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Serum Levels of Soluble Fas Ligand are Increased in Advanced Stage Melanoma

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The Fas ligand (FasL), a member of the tumor necrosis factor family, induces apoptosis in Fas-bearing cells. Accumulating evidence indicates that the Fas/Fas ligand (FasL) system is implicated in the pathogenesis of malignant melanoma, and that melanocyte FasL expression may contribute to melanoma immune escape. Membrane-bound human FasL can be converted to a soluble form (sFasL) by the action of a matrix metalloproteinase-like enzyme. Since FasL is expressed by melanomas, and in certain tumors sFasL levels have been reported to correlate with tumor mass, we evaluated the clinical significance of serum sFasL in malignant melanoma. An ELISA-based quantitation of soluble FasL was performed using sera from 51 patients with malignant melanoma (12 stage I, 12 stage II, 12 stage III, 15 stage IV) for which detailed clinical data including age, sex, clinical stage, and clinical course were available. Sera from healthy controls did not contain detectable levels of sFasL (<0.10 ng per ml), whereas those from melanoma patients did. In melanoma patients, serum sFasL increased with clinical stage (mean values: stage I: 0.09 ng per ml, stage II: 0.11 ng per ml, stage III: 0.14 ng per ml, stage IV: 1.59 ng per ml). There was a significant correlation between sFasL levels and clinical stage for stage IV patients. Although analysis of a larger number of samples from patients with stage I-III melanoma is required to determine if sFasL levels correlate with progression from stage I to III, these findings indicate that serum sFasL levels may be a useful indicator in evaluating disease activity in patients with malignant melanoma.

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Interleukin 10 Levels in Serum of Patients with Systemic Sclerosis

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Interleukin 10 (IL-10) activity is suggested in diseases of autoimmunological etiology (e.g., SLE and RA). There is scarcity of data concerning IL-10 in systemic sclerosis (SSc). Thus, the aim of our study was to evaluate IL-10 levels in serum of patients with SSc in comparison with skin score (according to Kahaleh *et al.*). Seventeen patients were included in our study (three males, 14 females; aged 31–70 y). Twelve of them presented the limited form (lSSc) and five the diffuse one (dSSc). The control group consisted of 19 healthy volunteers (18 females and one male, aged 16–60 y).

IL-10 levels were measured using ELISA method (Quantikine R&D Systems, INC, U.S.A.). The obtained results were statistically evaluated on the basis of Spearman rang correlation coefficient ρ. We observed: 1. higher IL-10 levels in SSc patients in comparison with the control group; 2. higher levels of IL-10 in dSSc patients than in lSSc group; 3. only in single patients there was some correlation between skin score (higher or lower) and (increased or decreased) concentration of IL-10 in serum during a 6-month follow-up period (I examination $\rho = 0{,}559$, $p < 0{,}02$, II examination $\rho = 0{,}385$, $p > 0{,}05$); 4. some correlation between IL-10 levels and skin score in lSSc group (I examination $\rho = 0{,}419$, II exam. $\rho = 0114$) and in dSSc group (I exam. $\rho = 0{,}486$, II exam. $\rho = 0522$) however, no statistically significant correlation was found ($p > 0{,}05$); 5. negative correlation $\rho = -0439$ between duration of the disease and IL-10 level in serum (the shorter the disease the higher IL-10 level in serum).

Our observations indicate the possible influence of IL-10 on the development of connective tissue in scleroderma probably resulting from diminishing INFγ and TNFα production. Another possible effect, similar as in SLE, can be connected with defect of inhibiting action of IL-10.

The obtained results point out IL-10 role in earlier stages of the disease. However, a 6-month follow up observations based on skin score and serum IL-10 levels, revealed that IL-10 is not a cytokine of crucial importance in evaluation of SSc activity.

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Circulating and Mucosal Immunological Defense of Atopic Dermatitis

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Atopic dermatitis (AD) is characterized by increased serum IgE levels with specificity for a wide variety of environmental allergens. Not only the IgE but also circulating immunoglobulins (IgG, IgA and IgM) might play an ethiological role in the pathogenesis of AD.

The aim of the study was to investigate the role of circulating (in the serum) and locally produced (in the lamina propria of mucous membranes) immunoglobulins involving IgE.

Diagnostic criteria of AD were based on the classification of Hanifin and Rajka. In 93 AD patients the classical immunoglobulins and IgE (total and specific) were measured in sera and in special cases (n = 11) in tear taken from AD patients.

Based on the increased serum total IgE levels, the patients were divided into extrinsic type and intrinsic type of atopy. Extrinsic type of AD (n = 79) showed hypersensitivity to inhalant and food allergens determined by the IgE test. Majority of the extrinsic type of AD showed only skin manifestations, while the rest of the patients (n = 11) showed in addition rhino-conjunctivitis. A comparative study was carried out to investigate the humoral antibodies not only in sera, but also in tear of these patients.The specific IgE test positivity in tear almost coexisted with elevated serum total IgE levels.

Results show that antibody positivity in tear is parallel with serum. The clinical significance and the practical aspect of the presented findings are that AD with mucous membrane involvement, e.g., rhinoconjunctivitis, can be diagnosed without blood test via IgE test performed from tear.

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Implication of Keratinocyte Derived Substance P in Allergic and Non-Allergic Cutaneous Inflammation

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Aim: To clarify the regulatory mechanism of substance P (SP) production by normal human keratinocytes in allergic and nonallergic cutaneous inflammation.

Methods: Normal human keratinocytes were stimulated with various drugs or cytokines and SP generated in the culture supernatant was measured by ELISA and mRNA expressions of preprotachykinin-a and NKR by KC were studied by RT-PCR and southern blot analysis.

Result and discussion: SP induced SP production by normal human KC in a dose dependent and autocrine fashion. Low dose glucocorticoid also induced SP production by KC. These results suggest that SP can be induced by KC in certain situations and might play an important role in allergic and stress induced nonallergic cutaneous inflammation.

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The Use of cAMP and IP_3, the Two Major Cellular Messenger Pathways, in Epidermal Cell Stimulation

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The cAMP pathway by protein G activation and the IP_3 pathway by Ca^{2+} flash are two major intracellular messengers that coordinate cellular metabolism. In these studies, we investigated the effect of associating a new molecule rich in GTP, a protein G activator, with synthesized IP_3 in order to stimulate human epidermal cells concordantly through these two mechanisms. The new molecule is extracted from zooplancton, and is composed of phosphorylated nucleotides (PN). The results of this association were compared with the effect of each molecule alone. Studies performed on cultured human A431 cells, keratinocytes and fibroblasts included immunostaining of representative proteins, electrophoresis and immunoblotting methods together with image processing. Cell proteins were extracted by PBS, SDS or pepsin limited digestion. Time and dose course response studies were performed on the different cell lines treated with PN, IP_3 or both. Our results showed that both PN and IP_3 increased cellular cAMP, and stimulated total and specific cellular protein synthesis. The maximum effect was reached after 24 h of PN application and within 10 h of IP_3 application. Moreover, filaggrin, keratins and Ki 67 immunostaining studies were performed on human skin organ culture as well as on reconstituted human epidermis. These studies showed an increase in epidermal cell differentiation, confirming the results obtained on cultured cells. Interestingly, the association of PN and IP_3 gave superior results and induced a higher increase in total cell protein with a higher stimulation of specific protein synthesis such as filaggrin, different keratins, fibronectin, collagen I and III. Furthermore, This effect was consistently present 10, 24 and 48 h after application of this complex. These studies demonstrate an interesting approach to cell stimulation by associating two pathways that provide stronger and longer-lasting cell activation.

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Evidence for Expression of the Neuropeptide Prohormone Convertases PC1 and PC2 in Human Dermal Fibroblasts

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The prohormone convertases PC-1 and PC-2 belong to a family of enzymes whose expression was originally considered to be strictly confined to neurendocrine tissue. Both enzymes are involved in processing of proopiomelanocortin (POMC) as well as other prohormones including prosomatostatin, proenkephalin or prodynorphin. Since the skin has recently evolved as an important target and generation site for the action of POMC-derived peptides such as α-melanocyte-stimulating hormorne (α-MSH), we wondered if human dermal fibroblasts express PC1 and PC2. Using RT-PCR and primers against PC-1, we detected specific transcripts of PC-1 in these cells. Immunofluoresecence studies revealed the presence of PC-1 and PC-2 in granular and organelle-associated intracytoplasmic structures sparing the nuclei. As determined by RT-PCR and Western immunoblotting, presence of PC-2 in fibroblasts was associated with expression of 7B2, a maturation factor and chaperone for PC-2. In order to assess the relevance of PC1 and PC2 expression, we measured the concentration of α-MSH by ELISA in culture supernatants and total cell lysates of fibroblasts after UVA irradiation, stimulation with interleukin-1 and the phorbolester PMA, the latter being known for its inducing effect of α-MSH in keratinocytes and melanocytes. None of these treatments resulted in detectable α-MSH levels in culture supernatants (detection limit 25 pg per ml). Likewise, although α-MSH could be demonstrated in marginal amounts intracellularly, these levels did not show any consistent change upon treatment as compared to unstimulated fibroblasts. Taken together, our results show expression of PC-1 and PC2 in human dermal fibroblasts. Expression of these enzymes in dermal fibroblasts suggests hitherto unrecognized functions other than secreting α-MSH.

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Cloning of a New Human Epidermis-Specific Serine Protease that may be Involved in Desquamation

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Degradation of desmosomes is important in desquamation. Several proteases may be involved in this process. We have previously isolated and characterized a chymotrypsin-like serine protease, SCCE, that seems to be involved in desquamation. We have now purified and cloned a new trypsin-like serine protease, also suggested to take part in desquamation, from human epidermis. We name this new enzyme stratum corneum tryptic enzyme (SCTE).

SCTE was purified from plantar stratum corneum, and its N terminal amino acid sequence determined. The whole coding part of the cDNA was cloned by several steps of PCR and sequenced. The cDNA was expressed using a eukaryotic virus-derived system. Protease activity was analyzed by zymography. To study the expression pattern of the *scte* cDNA, PCR was performed on cDNA from several different human organs.

The amino acid sequence deduced from the cDNA suggests that we have cloned a new trypsin like human serine protease. Recombinant protein could be harvested from culture media, indicating that the protein is exported from the cell. The recombinant protein showed protease activity after trypsin treatment, confirming that the cloned protein was a protease precursor. PCR screening of different organs showed the highest SCTE-expression in skin, and a low expression in kidney and brain.

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Identity of the Serpin-Enzyme-Complex Receptor with the FMLP Receptor

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Complexes of neutrophil-derived serine proteases with serpins are produced during neutrophil-mediated inflammatory reactions. Under septic conditions an increase of human leukocyte elastase – α_1-proteinase inhibitor complexes occurs in human serum. These complexes are known to express a neoepitope, which activates neutrophils via a postulated serpin-enzyme-complex-receptor (SEC-receptor). This receptor is also present on human monocytes and interestingly on hepatocytes. Stimulation of the SEC-receptor with a receptor specific peptide (FVYLI) results in acute phase protein synthesis in monocytes and hepatocytes.

In isolated human neutrophils FVYLI induced Ca^{++}-Influx as well as chemotaxis. We found that the Ca^{++}-Influx could be specifically desensitized by FVYLI and surprisingly by the bacteria-derived formylated peptide FMLP. Also the chemotactic response of neutrophils towards FVYLI could be suppressed by the FMLP receptor antagonist BocFMLP. In addition, receptor binding studies with 3H-FMLP revealed, that FVYLI competes with FMLP for the binding to the FMLP receptor of neutrophils. Furthermore, FVYLI proved to be an efficient chemotactic stimulus for monocytes and T-cells.

We conclude that the postulated SEC-receptor is not present on neutrophils, however, the functions of this receptor are mediated by the FMLP receptor. This opens a new view on the induction of acute phase protein synthesis, whereby exogenous mediators such as formylated peptides derived from bacteria as well as endogenous proteins such as elastase – α_1-proteinase inhibitor complexes might be able to induce acute phase protein responses during sepsis.

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The Molecular Chaperone 7B2 is Expressed in Human Skin Cells on RNA and Protein Level

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Proopiomelanocortin (POMC) is a polypeptide that can be cleaved into a number of neuropeptides with a broad range of activities, such as α-, β-, γ-melanocyte stimulating hormone (α-, β-, γ-MSH), adrenocorticotropic hormone β-lipotropin and β-endorphin. In the pituitary the prohormone convertases PC1 and PC2 mediate the controlled proteolysis of POMC. The generation of the potent immunomodulator α-MSH requires the proteolytic activity of PC2. Maturation and induction of the enzymatic activity of PC2 on the other hand depend on the presence of the neuroendocrine chaperone 7B2. Since POMC expression and α-MSH production recently have been detected in the skin, we investigated whether the mechanism of POMC-processing described for the pituitary also occurs in peripheral tissues. In a first step 7B2 expression was detected on mRNA level by RT-PCR in human monocytes, keratinocytes, microvascular endothelial cells, dermal fibroblasts and melanocytes, as well as in a panel of cell lines. Using western blot analysis we also demonstrated the presence of 7B2 protein in different human skin cell types. Among these were melanocytes, dermal fibroblasts, the melanoma cell line WM35, the monocytic cell lines U937 and THP-1, HMEC, HaCaT keratinocytes and sebocytes. Since we have previously reported that PC-1 is expressed in human skin cells, the finding that 7B2 also is expressed in the skin is an important step in the elucidation of extrapituitary α-MSH generation. Moreover these data will provide further insights in the control of α-MSH mediated immunmodulation.

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The New Molecule, Phytosterol Sulfate, Induces Human Keratinocyte Differentiation and is a Cell Membrane Stabilizer

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In epidermal biology, keratinocyte differentiation and stabilization of plasma membrane lipid bilayer are two important processes. In our scope of research, Phytosterol Sulfate (PS), a new molecule and homologue to Cholesterol Sulfate (CS) which is important for cell differentiation and epidermal barrier function, was developed from vegetables by a semisynthetic method. The effects of PS on human keratinocytes under membrane destabilizing conditions as well as the capacity of PS to modulate the differentiation of human epidermal cells and skin organ cultures were investigated. In addition, the effects of PS and CS were compared; analyses included light microscopy studies, immunoblotting experiments, and image processing. Cultured human keratinocytes and fibroblasts were pretreated with PS (or CS) followed by 10 μg per ml of SDS for 24 h. PS-treated cells demonstrated membrane protection, few SDS-induced morphological alterations, and only 10% loss of cells; by comparison, in untreated control cells, SDS-induced morphological changes were predominant and 60% of cell were lost. Using various concentrations and treatment times we studied the effects of PS on cultures of differentiating human keratinocytes and organ cultures of human skin. Light microscopy studies demonstrated that cultured keratinocytes slowed proliferation and became more differentiated after 1% PS application for 24 h. Specific immunostaining studies showed that PS enhanced the expression of differentiation markers such as involucrin and keratins 6 and 10 within 24 h; the maximum effect was found with 1% of PS after 48 h. These results were confirmed by immunoblotting studies of involucrin and keratins in treated and untreated keratinocytes and by studies on human skin in organ culture. Our results demonstrate the interesting role of the new molecule, Phytosterol Sulfate, in protecting keratinocytes from harmful conditions such as SDS insult, probably by enhancing their differentiation, membrane stability, and phospholipid content.

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Comparison Between the Dermoepithelial Junction of Human Sebaceous and Eccrine Glands by Immunofluorescence

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There has not been any previous systematic study of the dermoepithelial junction (DEJ) of skin adnexa. This is clearly important in relation to the investigation of disease processes at this site. Longitudinal cryostat sections from normal human scalp were stained with a panel of monoclonal and polyclonal antibodies (Abs) to the various components, including integrins, laminins, anchoring filaments, and anchoring fibrils of DEJ using standard immunofluorescence (IMF). In addition, some Abs with unknown antigens (Ags), such as LH24 and Tric 12/13 were also used.

In sebaceous glands, most of the Abs used showed a linear staining along the DEJ, except β1, which exhibited a chain-like pattern. Patterns of accentuation at the proximal portions of sebaceous lobules were seen with G71, 3E1, GB3, LH39 and 123. LH7.2, L3d, Ab to collagen VII triple helix (C7TH), LH24, and Tric 12/13 also had irregular subbasement membrane (BM) IMF in addition to linear BM staining. In eccrine glands, both the secretory and ductal regions were stained with the majority of Abs used. However, IMF could not be detected in the secretory portions with LH7.2, L3d, Ab to C7TH, LH24, and Tric 12/13.

In summary, the decrease of staining with Abs to anchoring filaments (LH39 and 123) at the distal sebaceous lobules and lack of IMF with Abs to anchoring fibrils (LH7.2, L3d, and Ab to C7TH) at the secretory regions of eccrine glands may contribute to their susceptibility to physical insults caused, e.g., by cryotherapy and radiotherapy. In addition, the unknown Ags for LH24 and Tric 12/13 may be associated with collagen VII because all of them have linear combined with sub-BM staining alongside the sebaceous glands and absence of staining at the secretory portions of eccrine glands.

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Normergic Cutaneous Delayed Type Hypersensitivity in Patients with Vitiligo

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Although the pathogenesis of the acquired depigmentation disorder vitiligo is still not completely clarified, many authors believe that an autoimmune reaction may play an important role. T-lymphocyte mediated immunity has frequently been strongly implicated in the pathogenesis of the disease. However, most studies applied *in vitro* testing of cell mediated immunity rather than *in vivo* measurements. Our study was undertaken to define the delayed type hypersensitivity (DTH) reaction in patients with vitiligo *in vivo*.

Cutaneous DTH was evaluated in normal pigmented skin of 109 vitiligo patients (29 men, 80 women) and in the depigmented skin of 27 of this group (five men, 22 women) using dermal application of seven common recall antigens together with a negative control. Individuals were considered hypoergic if the DTH sum score was ⩽ 5 mm in female or ⩽ 10 mm in male patients or if they responded to only one or two antigens.

The results of this study showed, that DTH in both depigmented and normal pigmented skin of vitiligo patients was normergic. The mean sum score was 10.2 ± 8.4 with an average of 2.3 ± 1.6 positive reactions in depigmented skin vs. a sum score of 12.4 ± 9.0 with an average number of 2.6 ± 1.6 positive reactions in normal pigmented skin. There was no statistically significant difference between results obtained in depigmented and normal pigmented skin of patients using the T-test ($p > 0.05$). Further evaluation of these data showed no significant correlation between various clinical parameters, such as clinical subtype of vitiligo, disease duration, age of onset, disease activity, family history, skin phototype and an aberration in cutaneous DTH (Pearson's correlation coefficient $r > [0.5]$).

In summary, in contrast to earlier reports, our *in vivo* studies of cutaneous DTH reactions revealed no clinically significant aberrant cellular immunity in vitiligo patients. The result of this study would support that the immune answer may be a secondary event in the pathogenesis cascade of vitiligo.

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Collagen Matrix Inhibits Effects of Oscillating Pressure in Human Dermal Fibroblasts

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Different culture systems have disclosed the importance of mechanical strength in cell behaviour. In order to standardize mechanical forces applied on human dermal fibroblasts, we choose to study the effects of controlled oscillating pressures.

Fibroblasts were seeded in monolayer on plastic or on collagen coated dishes and within a collagen matrix (dermal equivalent). Six days old cultures were placed in a pressure chamber where a sinusoidale frequency (0.01 Hz and 0.5 Hz) was applied through a computer controlled air compressor (from one to seven days, between 1.3 and 1.9 bars) during 20 min per day. Depending on experiment, fibroblasts were counted 24 h after the last treatment or lysed immediately after. In the last case tyrosine phosphorylation was analysed by SDS PAGE from cellular lysates followed by western blotting with an anti-phosphotyrosine antibody.

When fibroblast were grown on plastic, oscillating pressure (0.5 Hz) increased the cell number even with a single treatment while in the presence of collagen it did not. In parallel we observed an increase of phosphorylation of four proteins (about 12 Kd, 15 Kd, 24 Kd and 26 Kd). However, on collagen or within a collagen matrix these proteins were already phosphorylated and pressure did not increase this phosphorylation.

By this work we demonstrate that, depending on cell matrix interactions, oscillating pressure is able to modulate fibroblast behaviour. We need now to identify the phosphorylated proteins that seem to be related with cell matrix anchorage.

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The Influence of a Topical Corticosteroid on Short Contact Dithranol Therapy

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Since more than 80 y dithranol is a well established topical therapy for psoriasis. However, the perilesional and uninvolved skin often show irritation, which limits an optimal treatment with dithranol. The reports on the influence of topical corticosteroids on dithranol irritation are contradictory. The aim of the present study is to investigate the clinical and cell biological effect of clobetasol-17-propionate 0.05% in an ointment (CP) on dithranol treatment and perilesional irritation.

Eight patients with stable plaque psoriasis participated in this study. For 17 consecutive days 2% dithranol cream was applied on sites I and II. A 3th site (III) was left untreated. All sites consisted of lesional as well as perilesional skin of the back. After 1 h the cream was washed off, after which one site was treated with CP (site I), and one site with the vehicle (II). On day 17, three lesional and three perilesional biopsies were taken.

After 17 d the clinical scores of lesion I for erythema, induration and desquamation were significantly different compared to lesion II, showing a decrease to almost zero. The lesional skin of site I and II showed a significant different expression with regard to cycling epidermal cells and T-lymphocytes (dermal infiltrate). Comparing the lesional skin of site I and II with III a significant different expression was noted for epidermal cycling cells, T-lymphocytes, PMN, monocytes and macrophages. In the perilesional skin of site I and II a significant different expression was observed for epidermal cycling cells, involucrin, T-lymphocytes, PMN, monocytes and macrophages. The perilesional skin of site I and II compared with III demonstrated a significant different expression with regard to involucrin, T-lymphocytes, monocytes and macrophages. Remarkable, during a follow up period of 60 d we did not notice a rebound or relapse of lesion I.

The present study demonstrates a beneficial effect of CP on dithranol irritation. Moreover, the combination therapy of high dose short contact dithranol application with CP appears to be very effective in psoriasis.

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Normal Development of Desmosomal Plaque and Arm-Repeat Proteins in the Human Fetal Epidermis

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Desmosomal plaque proteins are membrane-associated cytoplasmatic proteins that can be subdivided into ubiquitous desmosomal constituents such as desmoplakin I and plakoglobin, and more cell-type specific proteins such as desmoplakin II and plakophilins 1 and 2. Along with β-catenin, plakoglobin and the plakophilins share variable numbers of repeats of a 42 aa-sequence originally identified in the armadillo gene product of Drosophila, and are therefore jointly referred to as "arm-repeat proteins". They are involved in the interaction of desmosomes and tonofilaments, while β-catenin promotes binding between actin and the adherens junction. Little is known about their normal fetal development.

The epidermal expression of desmoplakins and arm-repeat proteins was investigated in a total of 20 fetal (13–27 gestational weeks, GW) and four postnatal skin samples (2–50 wk of age). Biopsies were taken from back, scalp, and palmoplantar skin of aborted fetuses and from excessive tissue removed during abdominal and/or thoracal operative procedures, respectively. The study was approved by the local Ethical Committee. Samples were snap-frozen at –125°C, and immunofluorescence staining was performed with a panel of antibodies on microsections.

Plakophilin-1 was weakly expressed in the basal layer as of 16 GW, while intermediate and outer layers stained positive as of 13 GW. *Plakophilin-2* revealed a comparatively weak staining mainly at the basal layer as of 13 GW. *Desmoplakin*, *plakoglobin* and β-*catenin* were expressed in all layers throughout the fetal period. While plakoglobin and β-catenin showed equally intense staining in all epidermal layers, desmoplakin appeared to be expressed slightly less intensely in the basal layer. A comparison between two antiplakoglobin-antibodies (PG 11E4 and PG 5.1.7.2., respectively) consistently showed weaker staining of the latter in the basal epidermis.

Our study indicates that desmosomal plaque proteins are expressed early during fetal development. Sequential expression of individual proteins may point to their different role during epidermal differentiation. As evidenced by a recently described case of plakophilin-1 deficiency resulting in skin fragility and ectodermal dysplasia (McGrath J, Hoeger PH *et al*: *Br J Dermatol* 140:297–307, 1999), these structural proteins are involved in epidermal morphogenesis. Further studies are needed to study the molecular regulation of their synthesis.

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Reconstructed Mucosa Integrating Langerhans Cells

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HIV is commonly transmitted by sexual contacts through ano-genital or rectal mucosa. Langerhans cells, located in malpighian epithelia, are one of the first target cells of HIV since they express the main HIV receptor (CD4) and coreceptors (CXCR4 and CCR5). Until now, all three-dimensional *in vitro* mucosal models included only keratinocytes. In order to study HIV infection of Langerhans cells in conditions close to the *in vivo* situation, we performed a reconstructed mucosa including Langerhans cells.

The epithelium was composed of gingival or vaginal keratinocytes seeded on a de-epidermized dermis and grown in submerged culture for 2 wk, according to Green's method. Langerhans cell precursors, obtained after differentiation of cord blood-derived $CD34^+$ hematopoietic progenitors by granulocyte macrophage-colony stimulating factor and tumour necrosis factor-α, were introduced after 4–6 d of culture into the reconstructed epithelium.

Our model form a well pluristratified and differentiated epithelial structure, as shown by the immunostaining of specific cytokeratins : 4,10,13,14,16 and involucrin. Langerhans cells ($CD1a^+$ cells), were present in the suprabasal layer. The dermo-epithelial junction was visualized by using antibody anticollagen IV.

This reconstructed mucosal model allowed us to obtain similar characteristics to *in vivo* mucosal environment, and could be used for many studies including interactions with pathogenic agents (viruses, bacteria7), pharmacological, toxicological and clinical research.

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An Optimized Protocol for the Detection of Clonally Expanded T Cells in Cutaneous T Cell Lymphoma by Polymerase Chain Reaction and High-Resolution Electrophoresis

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Detection of T cell clonality by polymerase chain reaction (PCR) and high-resolution electrophoresis facilitates differentiation of early stages of cutaneous T cell lymphoma (CTCL) from benign T cell-rich dermatoses. However, data regarding the sensitivity of the electrophoresis techniques differ remarkably. The aim of the present study was to optimize our previously published heteroduplex-loaded temperature gradient gel electrophoresis (HD-TGGE) system as well as to determine sensitivity and lower detection limit before and after optimization.

With the standard protocol, the HD-TGGE detected clonal PCR product, achieved from the Jurkat cell line, down to 2 ng. However, the occurrence of slowly migrating single strands of the clonal PCR product reduced the amount of double stranded product which decreases remarkably the detection limit. To overcome single strand formation, the urea concentration in the gel and the temperature ramp for the heteroduplex formation were adapted. Furthermore, the temperature gradient in the gel was optimized. Application of the optimized protocol resulted in a tenfold improvement of the lower detection limit to 0.2 ng DNA in the clonal band. The sensitivity of the optimized HD-TGGE system was investigated by dilution experiments. Clonal Jurkat DNA PCR products diluted in nonclonal PCR products were detectable down to concentrations of 3–7%, whereas in PCR amplified mixtures of Jurkat DNA and nonclonal DNA the detection limit ranged from 10 to 20% Jurkat DNA. By dilution of Jurkat cells in nonclonal peripheral blood mononuclear cells, which corresponds best to the *in vivo* conditions, a lower detection limit of about 1% was observed.

Our findings demonstrate that optimized protocols for high resolution electrophoresis can enhance the detection of clonally expanded T cells. This is of particular importance for the differentiation between early stages of CTCL and benign T cell-rich dermatoses. However, our data also show that PCR and high-resolution electrophoresis do not have the high sensitivity previously assumed. Therefore, detection of clonally expanded T cells by these approaches reflects a higher frequency of tumor cells in CTCL than expected before.

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Immunohistochemical Expression of Bone Morphogenetic Protein-2 in Calcifying Epithelioma

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The mechanism of occurrence of ossification in calcifying epithelioma(CE) still remains ambiguous. To elucidate the pathogenesis of ossification in CE, the role of bone morphogenetic protein (BMP-2), which plays important roles in inducing ectopic bone formation both *in vivo* and *in vitro*, in CE was examined. Calcifying epithelloma (CE) (n = 20) were immunohistochemically studied using anti-BMP monoclonal antibody to differentially label the elements composing CE. In the normal skin including hair follicles, there was no BMP-2 expression. In all CE cases, BMP-2 was found exclusively in shadow cells and not in basophilic cells. In two cases of bone formation seen in CE, periosteal area and osteoblasts showed strong positive reaction, while bone trabeculum (bone matrix) showed no reaction. Our findings show that shadow cells positive for BMP-2 may be osteoprogenitor cells, and may indicate the preceding stage of bone formation in CE. BMP-2 plays an important role in generating bone formation in CE.

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Panniculitis-Like Subcutaneous T-Cell Lymphoma

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The panniculitis-like subcutaneous lymphoma is a new, recently identified, aggressive type of primary cutaneous cytotoxic CTCL. We observed eight cases (two children and six adults) of this lymphoma in the last 7 y. Clinically four patients showed disseminated lipomatous nodules and plaques, whereas in the remaining four cases inflammatory, burning lesions with frequent ulceration were observed. Most patients have had general symptoms and variable degree of peripheral blood abnormalities, such as an elevated ESR, LDH and a pancytopenia. In two patients bone marrow examination showed a myelodysplastic syndrome. Histologically these patients showed the specific involvement of the subcutaneous fat, with an angiocentric, intralobular and perilobular pleomorphic lymphoid and histiocytic infiltrate. Histiocytes were numerous and show variable degree of erythro-leucophagocytosis; in some cases (usually in inflammatory lesions) a strong granulomatous reaction was observed and the perivascular infiltrate involved also the mid and superficial dermis. Immunophenotypic analysis showed in all cases the expression of the CD3 pan-T cell antigen in association with the TIA-1 cytotoxic marker, whereas five cases were CD8+ and one showed the CD56 NK-like marker. Molecular analysis of the TCR-gamma showed a rearranged band in seven of eight patients. Five out of eight patients died for the disease, with a medium survival of 26 mo, two patients were in complete remission, respectively, 2 and 4 y after polychemotherapy and the last patient was recently treated by polychemotherapy. Our data confirm previous reports showing the peculiar clinico-pathologic, immunophenotypic and molecular features of this CTCL type.

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Primary Cutaneous Extranasal CD56+ Lymphomas

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The CD56+ primary cutaneous lymphomas are very rare and the clinico-pathologic, immunophenotypic and molecular characteristics of these patients ill defined. We observed 10 cases of this type of lymphoma in adults, nine man and one woman, ranging from 38 to 83-y-old. These patients usually showed multiple pink to red-brown or purpuric patches, subcutaneous nodules and plaques with possible ulceration localized at the back, at the arms or at the head. Nasal mucosa was involved only in two cases. Laboratory examinations and staging were normal, except for lymphocytosis (five patients) and platelet abnormality and myelodysplasia in one case. Patients, except the patient with myelodysplasia, were treated with polychemotherapy and radiotherapy, one patient also had autologous bone marrow transplantation, but the disease relapsed in short times with dissemination and leukemic involvement. Histology showed an angiocentric infiltrate formed by blastic cells (in four cases) or by a medium size pleomorphic lymphoid infiltrate involving the whole dermis, but sparing the superficial dermis. The immunophenotype showed the positivity of the CD56 NK-marker and the expression of cytoplasmic CD3 and CD45RA, whereas CD4+ and CD68+ (KP-1) were expressed in most cases and the CD2+ and CD7+ in four of 10. The TIA-1 cytotoxic marker was weakly expressed in three cases; the Ki-67 proliferation marker was strongly positive in most cases. PCR analysis of the TCR-γ showed no rearranged bands in all cases. In conclusion, due to the characteristic aggressive clinical course, histopathology and immunophenotype, we suggest that CD56+ primary cutaneous nasal-type NK lymphomas may represent a new "aggressive" CTCL entity, to be included in the EORTC classification for cutaneous lymphomas.

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Mast Cell Proliferation and Apoptosis in Cutaneous Mastocytosis –Immunohistochemical Study

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The aims of this study are to investigate the regulation of mast cell proliferation and apoptosis in cutaneous mastocytosis. We examined PCNA (proliferative cell nuclear antigen) staining, TUNEL method, and the expression of bcl-2 protein of mast cells in the lesional skin of 21 patients with cutaneous mastocytosis including eight of child-onset urticaria pigmentosa (UP), six of adult-onset UP, and seven of solitary mastocytoma using immunoperoxidase technique. Fifteen of 21 patients (71%) demonstrated proliferative activity of mast cells in the lesional skin (1–14% of mast cells) using PCNA staining. The percentage of PCNA-positive mast cells in child-onset UP was significantly higher than that of adult-onset UP. It may reflect the divergent clinical behavior between child-onset UP and adult-onset UP. Eight patients (38%) showed a few TUNEL positive mast cells infiltrated in the dermis (0.1–1.6% of mast cells). Twenty patients (95%) showed the expression of bcl-2 protein (1–83% of mast cells), which suppressed apoptosis in mast cells. These results suggest that apoptosis and its inhibition probably contribute the maintenance of the mast cell number in the lesional skin of cutaneous mastocytosis. UP in childhood is different from adult-onset UP with regard to mast cell proliferating activity.

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Immunohistochemical Evaluation of MIA-(Melanoma Inhibitory Activity) Antibody 2F7 in Malignant Melanoma

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We have recently shown that MIA is expressed in melanoma and melanoma cell lines but not in melanocytes and normal skin. Further investigations resulted in establishing an ELISA for detecting elevated MIA levels in sera of patients with metastasizing malignant melanoma.

To verify the specifity of MIA-expression in paraffin embedded material we examined 21 primary melanomas, 17 cutaneous and lymph node melanoma metastases and 20 nevi. Basaliomas, lymph nodes, angiosarcomas, T-Cell lymphomas and normal skin biopsies served as a control. For additional characterization of the MIA monoclonal antibody 2F7 we compared the immunohistochemical staining pattern with HMB-45 and S-100. Using the Labelled StreptAvidin-Biotin(LSAB)-technique all melanomas and metastases and 90% of the nevi were positive in S-100 and MIA staining. In contrast 95% of the primary tumors, 58% of the metastases and 61% of the nevi reacted with HMB-45. All controls were negative or normal, respectively.

Our results indicate that the MIA-antibody 2F7 is a sensitive and specific antibody in 100% of melanoma lesions. The advantage compared to HMB-45 and S-100 antibodies renders the 2F7 a useful tool also in immunohistological analysis of paraffin embedded sections of primary melanomas and melanoma metastases.

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Immunohistochemical Study of Vascular Endothelial Growth Factor and its Receptors Expression in Lesional Psoriatic Skin Before and after Treatment with Tazarotene

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Psoriasis is characterized by microvascular hyperpermeability and angiogenesis in the upper dermis, but the mechanisms responsible are unknown. Vascular endothelial growth factor (VEGF) is a 46-kDa glicoprotein driving angiogenesis or vasculogenesis in many human physiological and pathological conditions, such as embryonic development, wound healing, diabetic retinopathy, psoriasis and solid tumours growth. VEGF is produced by a variety of cells in, at least, four isoforms. The two smaller forms binds to one of the two receptors on endothelial cells: flt-1 and KDR. The aim of our study was to evaluate VEGF and its receptor (KDR)expression in lesional psoriatic skin before and after treatment with tazarotene (a retinoid acid receptor-specific retinoid). An immunohistochemical study on cryosections of lesional psoriatic skin was performed using antibodies to VEGF and KDR. Our results show, agreed with the literature, that in psoriatic skin VEGF and its receptor are overexpressed, suggesting that VEGF is a potentially important factor in the pathogenesis of psoriasis and providing further evidence that products of epithelial cells may regulate the inflammatory response. After eight weeks of treatment with tazarotene the expression of VEGF and its receptor was decreased in psoriatic lesions, suggesting the idea that therapeutic effects of tazarotene could act also through the downregulation of VEGF secreted by keratinocytes.

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Decreased Renal Blood Flow in Psoriatic Arthritis

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Psoriasis is a chronic inflammatory disease involving skin as well as joints in 10–20% of cases. Some patients with persistent lesions and longlasting course of the disease develop amyloidosis of the kidneys, especially those with pustular psoriasis, erythroderma and psoriatic arthritis.

The size of kidneys and their function was studied by both static scintigraphy using DMSA-99mTc and dynamic scintigraphy using DTPA-99mTc and Multispect-2-Siemens gamma-camera in 15 patients with psoriatic arthritis.

There was abnormal reciprocal kidney to kidney area ratio (<85%) in six cases, whereas in two patients both kidneys were significantly smaller (53% of patients). When six regions of both kidneys were analysed with respect to isotope uptake, it was decreased in 64% regions of the patients as compared to 2% regions in normal controls ($p < 0.01$). The analysis of vascular, filtration and excretion phases in dynamic scintigraphy has shown that renal blood flow was reduced more than 25% in two cases, marked decrease of total filtration index of both kidneys in two cases and one kidney in one patient were found, as well as there was reduced amplitude of filtration phases in three patients.

Our (results to) date have demonstrated that renal cortex is slightly impaired in more than half of the patients with psoriatic arthritis, who had both anatomical and functional renal changes.

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Epidermal Changes in Cryoblisters of Healthy Skin

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The exact level of the split occurring after cryosurgery has never been determined. Atrophic epidermal changes but not major dermal scarring is frequently seen after therapeutic doses of cryosurgery. In order to investigate the changes occurring in cryosurgery we performed immunofluorescence studies on cryoblisters using antibodies to antigens of the hemidesmosomal adhesion complex.

Single freeze cycles with liquid nitrogen of 5 and 15 s were performed on the forearm of a healthy volunteer. Excision biopsies of the cryoblisters were taken 24 h after cryosurgery and immediately snap frozen in liquid nitrogen. The experiment was performed twice. Microsections were exposed to mono-and polyclonal antibodies binding to $\alpha 6\beta 4$ integrin, laminin 5, collagen IV, collagen VII, collagen XVII and bullous pemphigoid antigen 1 and processed u sing a routine immunofluorescence technique.

The bullous pemphigoid antigen 1 was always detected at the roof of the blister and collagen IV always at the floor. Collagen XVII and $\alpha 6\beta 4$ integrin was mainly detected at the roof only faint staining was observed at the floor. Laminin 5 and collagen VII was mainly observed at the floor, in one experiment faint staining of laminin 5 was seen at the roof of the 5 and 15 s freeze blister and traces of collagen VII were observed in the same experiment in the roof of the 15 s freeze blister. These results demonstrate that the split in cryoblisters occurs at the level of the lamina lucida, however, there is no clear cut level of the split, perhaps the molecules are disrupted (collagen XVII, $\alpha 6\beta 4$ integrin, laminin 5). Longer freeze cycles my cause occasionally disruption of molecules in the lamina densa. The skin is split at similar levels in cryoblisters, suction blisters, bullous pemphigoid, acquired junctional epidermolysis bullosa and junctional epidermolysis bullosa. In some of theses conditions we observe atrophic skin lesions and presume that the level of the split contributes to the outcome as regards scarring.

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Cyclin A Overexpression in Nodular Basal Cell Carcinoma

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Cyclin A was shown to bind and activate cdk 2 in S phase and cdc 2 in G2 phase and to play an important role in the entry in M phase.

An increasing body of evidence suggests that cyclin A is also implicated in the regulation of S phase. We have previously reported a near doubling of S-phase duration in basal cell carcinoma as compared with normal human epidermis and produced data indicating that this phenomenon is associated with a decreased rate of DNA chain growth in the tumour cells. In this study, specimens of formalin-fixed, paraffin-embedded nodular basal cell carcinoma were analysed immunohistochemically for expression of cyclin A.

Of the 25 cases, 23 showed a strong nuclear expression of cyclin A in $9 \pm 1\%$ in the tumoral cells contrasting with a weak or absent expression in normal keratinocytes.

Cyclin A expression in the tumoral cells was observed mainly in cells in the S phase of the cell cycle. These results suggest a correlation between expression of cyclin A and the rate of DNA replication.

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The Notch Signalling in Hair Organization

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The Notch pathway is a cell-cell signalling system, involving interactions between a transmembrane receptor Notch, its transmembrane associated ligands, Delta or Jagged, and the secreted modulator Fringe. We investigated which different groups of partners among the four Notch, three Delta, two Jagged and three Fringe known proteins are associated to the three main phases of mouse hair vibrissa development? *In situ* hybridization showed that at the early embryonic stage corresponding to hair initiation, *Delta 1* is expressed as well as *Lunatic Fringe* in the cells forming the dermal papillae, while the surrounding dermal cells express *Notch 2*. A second and concomitant stage occurs in the embryonic epidermis. It leads to the segregation of the hair placode, which is delineated by the expression of *Jagged 2 and Lunatic Fringe*, whereas *Notch 1* is expressed both in the center of each placode as well as in the interplacodal epidermis. During the third phase, i.e., the organization of the embryonic hair peg into a hair follicle, as well as during the early anagen of the adult hair cycle, Notch 1, Jagged 2, Lunatic and Manic Fringe interact, Notch 1 being localized in the inner epidermal part of the developing follicle.

Our results suggest the double involvement in the skin of the Notch pathway between the cells located at the boundary of two expression domains. First, in the dermis, the formation or at least the stabilization of the dermal papillae appears to depend on the interaction between Delta1 and Notch 2. Second, in the epidermis, the expression of *Jagged 2* and *Notch 1* seems to pre-establish the further patterning of epidermal cells in two populations: those which will constitute the hair shaft and the inner root sheath, and those which will form the outer root sheath at the periphery. Lunatic Fringe, and to a lesser degree Manic Fringe appear as good candidates to modulate those interactions.

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Regulatory Sequences and Transcriptional Regulation of the Human MCP-4 Chemokine Gene (SCYA13) in Dermal Fibroblasts

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Monocyte Chemotactic Protein-4 (MCP-4) belongs to the β-chemokine family of cytokines. It is assumed to be involved in the accumulation of eosinophils characteristic for eosinophilic inflammatory diseases like atopic dermatitis, allergy and parasitic infections. Since MCP-4 is a candidate cytokine to be involved in the named diseases we were interested in the mechanisms governing gene regulation of this cytokine. We found upregulated MCP-4 mRNA expression in dermal fibroblasts upon stimulation with the proinflammatory cytokines TNF-α, IL-1α, IFN-γ, IL-5 and IL-4. Stimulation with either TNF-α or IL-1α combined with either IFN-γ and IL-4 increased MCP-4 upregulation. We isolated 1.4 kb of the immediate 5′ Promoter region where we found promoter elements potentially regulating MCP-4 gene expression and/or mediating the effects of anti-inflammatory drugs. Consensus sequences known to interact with nuclear factors like NF-IL6, AP-2, a NF-ΦB like consensus sequence γ-interferon-response elements and GAS elements are part of the MCP-4 promotor. INF-γ by itself induces MCP-4 and MCP-3 mRNA expression but not Eotaxin and RANTES mRNA. There is a striking correlation between the occurrence of a GAS element within the 200 bp promoter region proximal to the transcription start sites of the MCP-4 and MCP-3 promoters and the ability of INF-γ alone to upregulate MCP-4 and MCP-3 mRNA expression, which is not found in Eotaxin and RANTES. Reporter gene assays confirmed our hypothesis, that the 200 bp MCP-4 promoter region proximal to the transcription start mediates upregulation of gene expression by IFN-γ alone. Nuclear transcription factors are potential targets for intervention in skin-diseases. Our data point towards functional relevance and cell specific function of the identified regulatory promotor elements for MCP-4 gene expression.

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$P16^{INK4a}$ Inactivation and Telomerase Activity in Cutaneous T Cell Lymphoma (CTCL)

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Mycosis fungoides (MF) and its leukemic variant Sezary syndrome (SS) are the most frequent types of cutaneous T cell lymphomas (CTCL) and the aetiology of these diseases is quite unknown. To find out which genes are involved in the cancerogenesis of MF and SS, we investigated CTCL cells for the expression of DNA repair genes, cell cycle regulating genes, and telomerase activity. To determine the expression and activities of these genes we used Western and Southern blotting, PCR, RT-PCR, and PCR-ELISA. We found that the DNA repair genes hMLH1, hPMS1, hPMS2, hMSH2, GTBP were expressed in *CTCL* cells and that they had no obvious mutations. However, the cell cycle regulating genes $p16^{INK4a}$ and p18 proteins were not found in all four tested CTCL cell lines nor in the malignant T cells of CTCL patients. $p16^{INK4a}$ m-RNA was detected in all samples by RT-PCR with the exception of HUT78 and SeAx cell lines for which a deletion of exon 2 was found. Postranscriptional effects, may be due to subtle mutations and small deletions of $p16^{INK4a}$ or anomalies in gene regulation may be responsible for $p16^{INK4a}$ inactivation in the other cell lines and patients. Telomerase activity was detected in the cell lines and tested samples from patients. As telomerase activity was found in all samples, we conclude that telomerase activation is an early step in *CTCL* development. The inactivation of $p16^{INK4a}$ may be a further important step, as it has been shown that $p16^{INK4a}$ inactivation plus telomerase activation are sufficient for cell immortalization.

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Characterization of a 300 KB Region of Human DNA Containing a Domain of Type II Hair Keratin Genes

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A ≈300 kb contig of human genomic DNA was isolated via PCR screening of a human genomic P1 Artificial Chromosome (PAC) library using primer pairs derived from the hHb5 hair keratin cDNA. Two PAC clones were isolated, a 96-kb clone, termed PAC4, and a ≈220 kb clone, termed PAC5. These clones share a common 20 kb overlap encompassing the hHb5 gene. To this date, 11 type II hair and cytokeratin genes/pseudogenes have been isolated and identified/characterized from this contig. The hair keratin domain is flanked on one side by the cytokeratins K6b and K6hf and on the other side by K7. Inside this region, the genes encoding the human type II hair keratins hHb1, hHb3, hHb5, hHb6 have been identified/characterized, as well as the human ortholog of the mouse basic Hair Related Gene and three further, novel keratin gene/pseudogene loci. Similar to type II cytokeratin genes, the type II hair keratin genes consist of nine exons, eight of these exons being fairly conserved among each other. The size of the keratin gene loci on this contig range from ≈7–10 kb. Like their type I partners, the type II hair keratins appear to be clustered on the contig into groups of close amino acid homology, which reflect, in part, their regions of expression in the hair follicle. This contig encompasses approximately 45% of the human type II keratin domain on chromosome 12q13.

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Cloning and Overexpression of a *Propionibacterium acnes groEL* (Heat Shock Protein 60) Homologue and Purification of the GroEL Protein

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Heat shock proteins (Hsps) are dominant antigens of many bacteria and have been implicated in inflammatory processes. We hypothesise that Hsps are involved in inflammatory acne vulgaris. It was essential to confirm the presence of *P. acnes* heat shock gene homologues and to then clone and overexpress these genes to obtain sufficient protein for immunological investigation.

A fragment of a *P. acnes groEL* homologue was amplified by PCR using degenerate primers and used as a probe to screen a bacteriophage λ library of *P. acnes* genomic DNA to isolate the whole *groEL* gene. *P. acnes groEL* was cloned into the expression vector pET-30b and the GroEL protein overexpressed with a C-terminal histidine tag in *Escherichia coli*. The overexpressed protein was purified on a nickel-NTA agarose column.

The *groEL* gene of *P. acnes* encodes a protein of 544 amino acids with a predicted molecular weight of 56.8 kilodaltons and is not arranged in an operon with a *groES* homologue. *P. acnes* GroEL shows a high degree of homology to mycobacterial GroEL proteins (78% identity, 86% similarity to *Mycobacterium tuberculosis* GroEL2) which cause chronic inflammation in humans.

P. acnes possesses a GroEL homologue which has been cloned, overexpressed and purified and will now be used to determine its presence in inflamed follicles.

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Cloning and Preliminary Characterisation of the 5'-Flanking Region of Human Corneodesmosin Gene

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Corneodesmosin, a secreted protein restricted to the desmosomes and corneodesmosomes of the upper layers of cornified epithelia, is thought to have an adhesive function. Corneodesmosin gene, located 160 kb telomeric to HLA-C is a candidate in genetic susceptibility to psoriasis vulgaris. To understand differentiation-dependent and cell-type specific corneodesmosin expression we have cloned a 5'-flanking fragment of the human gene. Seven clones were isolated by screening a cosmid library from flow sorted human chromosome 6 (provided by RZPD at the Max Plank Institute) with corneodesmosin cDNA. A 4.2kb fragment flanking the 5'-end sequence of the cDNA was cloned and sequenced from clone ICRFc109cN1042QD2. Computer analysis predicted a promoter sequence with a TATA-box-like sequence (CATAAA) at position −44 from the 5′ end of cDNA, and a transcription start at position −13. As it is the case for several keratinocyte-specific genes, corneodesmosin promoter activity could be dependent on a proximal AP1 site (position −70). Sequence alignments revealed regions of high homology with the 5'-flanking region of the mouse gene, the 1350 proximal promoter region, and a fragment located between positions −2400 and −2850. To test the promoter activity *in vivo*, transgenic animals were generated with the 4.2 kb 5'-flanking fragment and the 5'UTR, linked to the *E. coli* -galactosidase gene. The progeny of several transgenic lines is used for analysis of transgene expression.

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Disruption of Collagen XVII (BP180) and Increased Expression of Keratins K16 and K17 Associated with a KRT10 Gene Mutation (R156H) in a Patient with Epidermolytic Hyperkeratosis

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It is now well established that epidermolytic hyperkeratosis (EHK), described clinically as bullous congenital ichthyosiform erythroderma (BCIE), results from the dominant negative effect of heterozygous point mutations in differentiation-specific keratin genes (KRT1, KRT10). However, the effects these mutations have on cellular processes are unknown. We do know that mutations weaken the keratin filament network, which then collapses under external pressure. However, we understand little of the effects of a keratin point mutation on epidermal cells and the surrounding tissues. Whilst investigating a patient with a blistering phenotype at birth for a suspected collagen XVII (BP180) mutation, it became apparent that a heterozygous point mutation in the KRT10 gene could disrupt collagen XVII expression.

The patient was born to nonconsanguineous parents with no family history of blistering. The phenotype was that of epidermolysis bullosa (EB) and a skin biopsy was taken for immunofluorescence investigation. Three basement membrane markers were normal (laminin 5, collagens IV and VII) but staining for collagen XVII and α6 integrin showed accumulation in keratinocytes. However, no mutation was found after screening the collagen XVII, laminin 5, and α6 integrin genes. The phenotype changed to that of EHK at 2-y-old. Routine screening for keratin mutations identified a common heterozygous point mutation in K10 (R156H). Immunofluorescence investigations of K16 and K17 expression showed that these were increased dramatically. We conclude that this common keratin 10 mutation (R156H) has effects on the epidermis over and above that of filament collapse, and can effect the structure and expression of other keratinocyte proteins such as collagen XVII α6 integrin and K16.

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Compound Heterozygosity for Splice Site (267-1G→A) and Missense (P1699L) Mutations in COL7A1 Gene Results in Pretibial Dystrophic Epidermolysis Bullosa

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Pretibial epidermolysis bullosa (PEB) is a rare form of localized dystrophic epidermolysis bullosa characterized by late age onset, blistering and hypertrophic scarring in pretibial area, and nail dystrophies. Most PEB cases show autosomal dominant inheritance and a glycine substitution mutation in COL7A1 gene has been identified in a PEB kindred. We report a 29-y-old man affected by PEB displaying abnormal anchoring fibrils (AF) and reduced immunostaining for type VII collagen. The patient was shown to be a compound heterozygote for novel mutations affecting COL7A1 gene. The first mutation is a G→C transversion at position −1 of exon 3 (267-1G→C). Mutation 267-1G→C alters the correct splicing of COL7A1 premRNA giving rise to two major aberrant transcripts, one carrying the skipping of exon 3 and the other resulting from the usage of a cryptic splice site 71 bp upstream of the normal splice site. Either way, aberrant splicing leads to premature termination codon (PTC) formation. The second mutation is a C→T transition which converts a conserved codon for proline at the Y position of a Gly-X-Y collagenous repeat into a codon for leucine (P1699L). The localized PEB phenotype of our patient appears therefore to result from the combination of PTCs on one allele and a missense mutation on the other. Mutation P1699L represents the first example of a proline substitution within COL7A1 and can be added to the list of recessively inherited glycine and arginine substitution mutations described in COL7A1. Substitution of a proline in a putative hydroxylation site, such as the Y position of a Gly-X-Y repeat, may reduce the stability of the triple helix and/or interfere with collagen VII supramolecular assembly, explaining the abnormal appearance of AF. Finally, our findings underline the interest of molecular diagnosis in sporadic PEB in order to distinguish between *de novo* dominant mutations and recessive inheritance.

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Gene Regulation by Mechanical Forces in Human Dermal Fibroblasts

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The influence of mechanical forces on skin metabolism is still a poorly understood phenomenon. As *in vitro* model, primary human dermal fibroblasts are cultured in 3D collagen lattices that are either restrained (high tension) or allowed to contract (low tension). Gene regulation was studied by Northern blot hybridization, yielding differences in expression of several extracellular matrix proteins, matrix-degrading MMP-1, certain surface molecules and mediators, and cytoskeletal structural proteins.

A systematic analysis of many defined genes was performed employing the ATLAS array which showed differential regulation of genes encoding transcription factors, stress response and apoptosis genes and cell surface antigens. Some of these genes have been studied in more detail, and it became clear that mechanical forces lead to inhibition of apoptosis and induction of mRNA specific for fibrogenic cytokines, like TGF-β1, -β3 and CTGF.

In order to identify unknown genes which are regulated by mechanical tension in the described experimental system, we employed a combination of subtractive cDNA hybridization and subsequent amplification of unique sequences. So far, we obtained several sequences of unknown genes, some of which have previously been isolated from mechanically strained tissues, such as pregnant uterus or fetal heart. Their differential regulation was verified by Northern blot analysis. We then subcloned new gene sequences, and carried out *in situ* hybridization and Northern blot analysis using human skin and scars. One of these sequences is expressed in fibroblasts, keratinocytes and endothelial cells, showing strong upregulation in early scar formation in skin.

Using this approach, we hope to identify genes which play a role or serve as markers in pathological conditions of tissues under stress.

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Interaction of Five Gene Polymorphisms at Psoriasis

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Associations among five gene polymorphism interactions and clinically manifested psoriasis were studied. All studied gene polymorphisms (endothelin-1-ET-1, transporters associated with antigen processing-TAP1, tumor necrosis factor β-TNFβ, angiotensin I converting enzyme-ACE, advanced glycosylation end product–specific receptor-RAGE) were analyzed for their possible participation in the MHC class I cell surface antigen presentation to CD8+ T-cells. Polymerase chain reaction (PCR) protocols were used to characterize the gene polymorphism. In the study, 153 patients with psoriasis were included. In psoriatic patients, the disequilibrium was found for coincidence of TNFβ with TAPl-333 (Ile→Val at position 333) ($p = 0.016$) as well as with TAP1–637 (Asp→Gly at position 637) ($p = 0.035$). TNFβ was in disequilibrium with (ACE) polymorphism ($p = 0.036$). ACE was observed to be in tight disequilibrium with TAP1-333 ($p = 0.009$) but not with TAP1-637 ($p = 0.133$). The borderline significance of disequilibrium was found for ET-1 and RAGE gene polymorphisms ($p = 0.07$).We proved genetic disequilibria in double genotype coincidence between HLA I class molecule polymorphisms at 6p 21.3 (TNF, TAP1), between RAGE (6p21.3) and ET-1 (6p24-p23), between ACE (17q23) and TNFβ and between ACE and TAP1-333 polymorphisms in psoriatic patients. These disequilibria reflect interactions among gene determinants of MHC I antigen presentation which could participate in manifestation of disease.

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Apoptotic Pathways in Drug Sensitive and Drug Resistant Melanoma Cell Lines

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Recent studies showed that chemotherapeutic agents can kill susceptible cells through induction of apoptosis. Thus, drug resistance of tumor cells may be mediated by modulation of apoptotic pathways. We studied mechanisms of drug induced cell death and apoptosis in the drug sensitive melanoma cell line MeWo and MeWo derived sublines resistant to four different drugs (cisplatin, etoposide, fotemustine, vindesine). Expression of different proteins known to be involved in apoptosis signaling (e.g., CD95/FAS, TRAIL-R1–4, caspases) was analysed on RNA-(RNase protection assay) and protein-levels (Western blot, FACScan). Cisplatin and etoposide were found to induce apoptotic cell death as indicated by (I) drug induced DNA fragmentation and (II) the antagonistic effect of inhibitors of apoptotic caspases (e.g., zVADfmk). MeWo cells constitutively express TRAIL-R2, while CD95, TRAIL-R1, -R3, -R4, and TRAMP could not be detected. Exposure to cisplatin and etoposide induced expression of CD95 and slightly increased the amount of TRAIL-R2 mRNA. Processing of the caspase substrate PARP could be detected after long time exposure to drugs (about 96 h). However, this was not preceded by activation of caspase-8. Only cells resistant to cisplatin showed reduced TRAIL-R2 mRNA levels. In comparison to parental cells in all drug resistant sublines mRNA levels of other death receptors and caspase-8 were not changed. The results indicate that at least resistance to cisplatin may be mediated by alterations in death receptor expressions. Further studies are needed to completely understand the apoptotic pathway involved and to identify relevant caspase(s).

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Transcriptional Regulation of the Melanoma Cell Adhesion Molecule Gene by Sp1, AP-2, cAMP and SCF/c-Kit Signaling

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The melanoma cell adhesion molecule (MCAM) was identified as a human melanoma-associated antigen that increases in expression as tumors increase in vertical thickness and begin to acquire metastatic potential. The mechanisms for up-regulation of MCAM during melanoma progression are, however, still poorly understood. In this study, we show that MCAM expression is tightly regulated at the transcriptional level.

Using a combination of CAT reporter assays and semiquantitative RT-PCR, we observed that cAMP significantly increases transcription of MCAM in nonmetastatic melanoma cells. In metastatic cells, transcription of the gene was constitutive and could not be further increased by cAMP. On the other hand, MCAM promoter activity was impeded upon treatment with phorbol esters or in the presence of SCF, a phenomenon which was PKC-dependent. Promoter-deletion studies demonstrated that the first 196-nt of the MCAM promoter region are sufficient to get full expression in metastatic melanoma cells. This fragment contains five binding sites for the transcription factor Sp1. DNA mobility shift experiments showed direct binding of Sp1 to the promoter and we could demonstrate that Sp1 is sufficient to drive constitutive MCAM expression in metastatic melanoma cells. In nonmetastatic cells, however, MCAM expression is repressed and it has been postulated that the transcription factor AP-2 could mediate this repression. Although downregulation of MCAM expression in the presence of AP-2 could be confirmed, our results strongly suggest that the role of AP-2 might be indirect, indicating that additional regulatory factors may be required in the negative control of the MCAM gene.

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Cre-Recombinase Mediated Induction of Urokinase Plasminogen Activator: a Novel Genetic Switch

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Urokinase plasminogen activator (uPA) is the predominant plasminogen activator present in keratinocytes and normal epidermis. uPA plays a major role in a number of physiological conditions in which extracellular matrix degradation is required for migration and tissue remodelling, e.g., wound healing. To facilitate study of this pathway *in vitro* and *in vivo* we have developed an inducible expression system utilising the bacteriophage cre-recombinase system.

Full length murine uPA cDNA, minus its ATG start codon, was cloned directly downstream from a loxP flanked transcriptional stop sequence (TSS) and a replacement ATG sequence was placed immediately. upstream of the TSS. This plasmid was termed TSS-uPA. cre-driven recombination splices the TSS plus one of the two loxP sites from the sequence, allowing full length uPA to be expressed from the upstream in-frame ATG. To test the efficiency of this strategy, TSS-uPA was cloned into the pcDNA-3 mammalian expression vector and transfected into CHO cells which preliminary studies had shown express only a low background of uPA. Analysis of transient transfection supernatants by indirect colorimetric assay using the plasmin substrate S-2251 showed a marked increase (>300-fold) in plasminogen activation when the TSS-uPA construct was cotransfected with a cre expression vector compared to the TSS-uPA construct alone ($n = 3$). This increase was completely abolished by the addition of amiloride confirming that the increase is due to functional uPA. Finally, PCR analysis of doubly transfected cells showed a novel band confirming that recombination had occurred.

The ability to inducibly express important biological factors, such as uPA, represents a powerful tool for the study of these factors *in vitro* and *in vivo*. The well characterized cre-recombinase system is well suited to this role and should prove useful in many areas of skin biology.

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The Expression of Retinoic Acid Receptor α, Retinoid X Receptor α and Thyroid Hormone Receptor α is Reduced in Psoriatic Lesions as Compared to Nonlesional Skin

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Retinoic acid, vitamin D3 and triiodothyronine are all involved in the regulation of keratinocyte proliferation and differentiation, i.e., processes that are disturbed in lesional psoriatic skin. The effects are mediated via nuclear hormone receptors; viz. the retinoic acid receptors (RAR-α, -γ), the vitamin D3 receptor (VDR), the thyroid hormone receptors (TR-α, -β) and the common heterodimer partners, the 9-cis retinoic acid receptors RXR-α and -β. By using a quantitative real-time PCR the mRNA expression of these receptors and three different house-keeping genes (cyclophilin, GAPDH and β-actin) was studied in psoriatic skin. Since the expression of the house-keeping genes was constantly 2.7–4.4 times higher in lesional as compared to non-lesional skin one of them (β-actin) was used to normalize the expression of the nuclear receptor genes. Thus, the mRNA levels of RARα, RXRα and TRα in lesional skin were found to be decreased by 58, 70, and 75%, respectively, while RARα, VDR, and TRβ were not significantly altered. As a consequence, the ratio of RXRα:RARγ, which was 3.3 in nonlesional skin, decreased to 1.3 in lesional skin. The results suggest that retinoid and thyroid hormone signaling systems are altered in lesional psoriatic skin.

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HLA DR 4 and the Increased Risk of Melanoma in a Celtic Population. A Study of 108 Patients

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Controversial data have been reported about HLA alleles and susceptibility to melanoma. Several reports agree on the role of Major Histocompatibility Complex (MHC) molecules in the immunosurveillance against tumor. MHC class I molecules are involved in the effector phases, while MHC class II molecules are important in the induction of the immune response. Individuals of Celtic ancestry are claimed to be at a greater risk of skin cancer than non-Celts. Many factors have been discussed to explain that, including skin phototype, pattern of sun exposure, and other ill-defined factors. An association between HLA DR4 and melanoma has been described in southern Australia and recently the higher frequency of HLA DR4 phenotype in Celts from Wales was assessed. To confirm these data, we conducted a systematic study in patients with melanoma, in a Celtic population from Brittany.

Frequency of HLA DR4 of 108 adult patients with melanoma from our area in west Brittany of Celtic origins was compared with frequency of HLA DR4 of 150 unrelated bone marrow donors originating from the same region. HLA typing were performed by PCR-SSP and according to WHO nomenclature. 216 chromosomes were studied from 108 melanoma patients and 300 from 150 controls.

No difference was noted in the two populations studied. The rate of HLA DR4 in melanoma patients was 18%, similar to the rate of 19,6% in the general population of Celtic origins in our area ($p > 0,05$).

Our data do not confirm previous reports, which emphasized the higher prevalence of HLA DR4 in melanoma patients in a Celtic population. Furthermore in our area, the high incidences of recessive diseases confirm the low melting rate of our Celtic population. To our opinion, HLA DR4 do not give a higher genetic risk to develop melanoma

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Analysis of Psoriasis-Relevant Gene Expression by RT-PCR/Differential Display Comparison Between Psoriatic Involved Skin and Normal Oral Mucosa

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The etiology of psoriasis is still unknown, although there is an overwhelming evidence that the disease is dominated by autoimmune-like pathomechanisms. Therefore it has been hypothesized that psoriasis-related auto-antigens could exist as a possible target especially for T-cellular reactions. Moreover, psoriasis affects the mucosa of the lips and tongue only in rare cases, whereas psoriatic involvement of the buccal and pharyngeal mucosa is an exceedingly rare event. Given these facts, we put up an experimental model system comparing psoriatic involved skin and normal buccopharyngeal mucosa by a RT-PCR/differential display strategy in order to identify proteins, which may be of crucial pathogenetic relevance for psoriasis.

For the initial RT/PCR we used a set of oligo-T and arbitrary primers recently described by other authors (*Nucleic Acids Res* 21:4272, 1993). Differential display was performed by nondenaturing, horizontal PAGE with subsequent nonradioactive detection of cDNA bands by an optimized silver impregnation. Differentially displayed cDNA species were extracted from the gel, PCR-reamplified, subcloned and sequenced.

We found more than 70 cDNA species to be displayed in psoriasis, but missing in oral mucosa, with until now unknown DNA sequences. The data were communicated to GenBank and received accession numbers, such as AF117823–5, AF119788, 125378–125380, 126039–44, AF126950.

In conclusion, we are presenting a novel research model aiming at the identification of gene products which might be critically involved in the etiopathogenesis of psoriasis with regard to (a) the epidermal hyperproliferation, (b) the vascular-inflammatory compartment as well as (c) putatively involved auto-antigens. The further characterization and identification of the sequence data is an ongoing process.

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Characterisation of Expressed Genes in Normal and TNF-α Stimulated Human Epidermal Keratinocytes

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Using *in vitro* models and Serial Analysis of Gene Expression (SAGE), changes in gene expression patterns were analysed after stimulation of keratinocytes by TNF-α, a cytokine that is thought to play an important role in abnormal epidermal differentiation as seen in psoriasis. SAGE is based on two principles: first, a 10-bp nucleotide sequence or "tag" is sufficient to uniquely identify a transcript, and second, concatenation, cloning and sequencing of these tags allows rapid, simultaneous analysis of thousands of transcripts, and gives information about relative abundance. 25964 tags have been sequenced, possibly representing more than 10000 genes. Several functional classes of genes have been identified, irrespective of TNF-α stimulation. The most abundantly expressed genes are those involved in metabolic pathways and protein synthesis. Furthermore, genes that are involved in keratinisation and the establishment of the epidermal barrier are all expressed at high levels, as well as genes that encode antimicrobial proteins and protease inhibitors, which are believed to play an important role in host defense. Some genes of the latter group were found to be induced by TNF-α. Several pro- and anti-apoptotic genes were also expressed in both libraries. Differential expression was confirmed by Northern blot hybridization. Moreover, SAGE has allowed us to identify previously unknown genes that are differentially regulated by TNF-α.

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Expression of Sarco/Endo-Plasmic Reticulum Ca^{2+}-ATPase Type 2 Isoform in Normal Skin

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Recent work has established that mutations in the *ATP2A2* gene cause Darier's disease. *ATP2A2* encodes the type 2 isoforms of the Ca^{2+} transporting sarco/endo-plasmic reticulum pump (SERCA2). The aim of our study was to determine the expression of SERCA2 in normal skin.

Eighteen samples of normal skin were tested with the mouse monoclonal antibody NCL-SERCA2 (Novo Castra laboratories) using a standard ABC peroxidase technique (Vectastain ABC Kit). Non-immune mouse IgG_1 served as a negative control.

SERCA2 was expressed in the epidermis in all samples. Expression was most marked in the basal epidermal cells but 16 samples also demonstrated patchy expression of SERCA2 in supra basal keratinocytes. Additionally, SERCA2 was expressed in the outer root sheath of hair follicles, the basal layer of sebaceous glands, basal and secretary cells of eccrine glands, pilar muscle and blood vessel walls.

This is the first report documenting the distribution of SERCA2 in normal skin. Our results support the hypothesis that *ATP2A2* is an important "house keeping" gene involved in the regulation of cellular differentiation and proliferation in the epidermis.

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Identification of Vitamin D Responding Genes in Psoriatic Skin by Differential Display

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The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) regulates gene expression through the vitamin D receptor and specific sequences in the promoter region of target genes. $1,25(OH)_2D_3$ and analogues of vitamin D have been shown to improve psoriasis, characterized by immune activation, increased proliferation and abnormal differentiation of epidermal keratinocytes. However, the mode of action of vitamin D in psoriasis is unknown. Thus, it is not known which genes are regulated when the skin is treated with $1,25(OH)_2D_3$ or analogues of vitamin D. Only few vitamin D responding genes have been identified in the skin or in cultures of human epidermal keratinocytes. In order to identify new targets for the actions of D-vitamins in human skin the mRNA differential display technique has been used for the identification of genes differentially expressed in psoriatic skin treated with the vitamin D analogue calcipotriol.

Experiments were conducted on RNA isolated from biopsies from calcipotriol- or placebo-treated psoriatic skin. Using the differential display procedure with 24 different primer combinations, 24 differentially expressed cDNA fragments were isolated from psoriatic skin. By cloning and sequencing the fragments and then by searching gene banks for sequence similarity, it was found that six of the fragments had a high similarity with known genes. Currently, work is in progress to verify these findings and elucidate the biological significance.

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Polymerase Chain Reaction Analysis of T-Cell Receptor γ Gene Rearrangements in Cutaneous B-Cell Lymphomas

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Polymerase chain reaction (PCR) analyses of T-cell receptor (TCR) gene rearrangements in B-lymphoid neoplasms have shown lineage infidelity and double rearrangements involving both immunoglobulin heavy chain (igH) and TCRγ genes. In particular, a recent study (*Br J Dermatol* 137:673–679, 1997) has demonstrated that 30% of cutaneous B cell lymphomas (CBCL) show clonal rearrangements of the TCRγ and the authors concluded that TCRγ gene rearrangement cannot be used as a T-cell lineage marker. In this investigation, we have tested for clonal TCRγ rearrangements a panel of immunophenotipically and genotipically well characterized CBCL. Diagnoses were established by routine histology and immunohistochemistry. Each of these cases displayed clonal igH gene rearrangement. Polymerase chain reaction (PCR) analysis of the TCRγ rearrangements followed by high-resolution polyacrylamide gel electrophoresis was utilized for detection of clonal TCRγ rearrangements. In our investigation, none of the cases of CBCL investigated by PCR showed the presence of clonal TCRγ gene rearrangements. These data indicate that double rearrangements involving both igH and TCRγ genes are not a feature of CBCL and confirm that TCRγ gene rearrangement can be used as a T-cell lineage marker of cutaneous lymphomas.

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Investigation of Tumor Necrosis Factor-α Induced Tumor Rejection in Mice with Severe Combined Imunodeficiency

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We have genetically modified murine and human tumor cell lines (HeLa human cervical cancer, B16 murine melanoma, HT168 human melanoma) that produce high quantities of human tumor necrosis factor (TNF)-α, while preserving their original morphology and growth characteristics. These cell lines show reduced tumorigenicity, the reduction being proportional to the production of TNF-α. The growth restriction was not due to the presence of TNF-α by paracrine mechanism, because the cells are TNF insensitive, moreover normal mice injected with cells modified with the trans-membrane form of TNF-α developed immunity towards the original tumor cells as well. Our goal was to identify immunocytes responsible for the phenomenon, so we repeated the experiments in BALB/C mice immunosuppressed with antithymocyte sera. In the T-cell depleted mice native tumor cells grew well, unlike their TNF-producing modified variants. Tumor growth did not occur however, in mice injected on one side with the TNF-producing cells, on the other side with the native nonproducing cells. This rejection seems to be T-cell independent. In mice with severe combined immunodeficiency (SCID) lacking both mature T and B cells growth of TNF-producing cells was significantly slower than their wild type variants, but engraftment was always successful. In SCID mice the injection of mitomycin-treated TNF producing cells prevented the engraftment of either TNF-producing or native tumor cells of the same cell lineage. The same pretreated mice could be engrafted successfully with cells from another tumor group though. Activation of immunocytes present in SCID mice (NK, LAK, CIK or myelocytes responsible for nonspecific immunity) and their specific cytotoxic abilities were investigated with TNF-producing cells. Our results indicate that membrane-bound TNF and its soluble variant trigger different signals in these cells.

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Efficient Expression of the Tumor-Rejection Antigen MAGE-3 in Human Dendritic Cells Using an Avian Influenza Virus Vector

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Several studies suggest the crucial role of dendritic cells (DC) for the induction of antitumor immunity. For this purpose DC must be modified to express tumor-rejection antigens, e.g., genetically. Virus vectors constitute an efficient system for gene delivery. Since influenza viruses efficiently infect DC without changing their antigen presenting capacity we used a recombinant avian influenza vector to introduce the tumor-rejection antigen MAGE-3 into mature monocyte-derived DC. The polymerase I system was used to generate recombinant influenza vectors encoding MAGE-3 or the Markerprotein GFP. More than 90% of the DC could be infected with the GFP control as analyzed by FACS. Next we tested the influenza vectors encoding MAGE-3. Efficient expression of the MAGE-3 protein could be demonstrated by immunoblotting as well as by intracellular staining. Importantly the transduced DC retained their characteristic phenotype and their potent stimulatory capacity in allogenic MLR. Experiments are now in progress to test the ability of these transduced DC to elicit specific CTL responses.

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Linkage Disequilibrium Analysis of the Chromosome 4q Psoriasis Susceptibility Region Supports a Founder Effect in Irish Families and Narrows the Putative Gene Location to a 1 Megabase Interval

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The genes responsible for the familial clustering of psoriasis are not known. However, genome-wide linkage scans have implicated several chromosomal regions, including a 4q locus originally identified in a largely Irish family collection. Our strategy has been to use linkage disequilibrium (LD) analyses to detect founder haplotypes underlying the Irish 4q susceptibility effect, thereby confirming the original linkage and fine-mapping the aetiological gene. A total of 58 parent-offspring trios were ascertained through an Irish proband with psoriasis (50% male; mean age of onset 14.7 y). Together with seven of the original families, they were genotyped for 22 highly polymorphic microsatellite markers spanning the region of linkage (mean marker density of 1 per 250 kb). LD was sought using a multilocus transmission disequilibrium test (GENEHUNTER v2). In the 1 Mb interval bounded by CHLCGAAT1B3 and D4S1535, we found clear evidence for skewed transmission of certain 3-locus (14 of 15 transmissions $p = 0.0008$) and 4-locus ($p = 0.008$) haplotypes to affected offspring. These data provide support for a 4q mutation contributing to psoriasis susceptibility in the Irish population, and localise that mutation to a 1 Mb region.

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Dystrophic EB: Optimization of Collagen VII Microscale Detection Systems for Geno-Phenotype Correlations and Gene Therapy Approaches

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Recessive dystrophic epidermolysis bullosa (RDEB) is one of the most severe inherited bullous skin disorders. RDEB patients suffer from loss of dermo-epidermal adhesion due to mutations in the COL7A1 gene which encodes for collagen VII, the major component of the anchoring fibrils. So far, the lack of therapy for patients with severe RDEB provides an impetus to develop gene therapy approaches. We performed mutation screening by heteroduplex analysis and automated DNA sequencing of RDEB patients. Two novel COL7A1 frameshift mutations were identified in a patient with RDEB mutilans. The patient is a compound heterozygous for a 1 bp deletion in exon 5 (638delG) and a 1 bp deletion in exon 51 (4871delC), both mutations lead to a premature termination codon. For determination of collagen VII protein expression in the patients cells and for gene therapy approaches, we developed a new, highly sensitive polyclonal antibody against recombinant epitopes in the NC1-domain of collagen VII. With this new antibody the patient was shown to be completely collagen VII-deficient and therefore he is an optimal candidate for gene therapy approaches. For the analysis of gene-targeted collagen VII-deficient cells, the new antibody was employed in indirect immunofluorescence staining, Western blot analysis and a cell-ELISA. With these assays it was possible to detect as few as 1% collagen VII-producing keratinocytes in a cell mixture. Further, these assays allowed quantitation of small amounts of collagen VII synthesized by fibroblasts for the first time: only 1500 normal fibroblasts or 5 ml medium were required for collagen VII detection by Western blot analysis. Thus, microscale detection of collagen VII is possible and useful for collagen VII analysis in RDEB patients cells and in gene-targeted keratinocytes.

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Linkage Analysis of Two Families with Both Skin and Neurological Disorders

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Several diseases have previously been described in which a skin disorder is found to cosegregate with neurological abnormalities, for example erythrokeratodermia and ataxia and palmoplantar keratoderma and Charcot-Marie-Tooth disease. Here we describe the phenotypic and genotypic characteristics of two families who have inherited disorders affecting both the skin and the nervous system. These disorders cosegregate in the first family but are distinct in the second.

A Scottish family presents a dominant disorder characterised by focal palmoplantar keratoderma (PPK), nail dystrophy and motor and sensory neuropathy. Five candidate regions have been excluded from this family using microsatellite markers flanking each region, which were analysed on a LiCor automated sequencer. These regions include the connexin gene cluster on 1p34-p35, keratin gene clusters and desmosomal associated proteins such as BPAG1 and periplakin.

A consanguineous Bedouin family has recently been identified in which two unrelated autosomal recessive disorders, exfoliative ichthyosis and encephalopathy are segregating. In these patients the ichthyosis appears shortly after birth with peeling of skin on the palms and sole, and scaling over the entire body. The encephalopathy causes seizures and mental retardation. Fifteen epidermal candidate regions including the type I and II keratin clusters at 17q12-q21 and 12q13, cadherin gene clusters at 16q22 and 18q12 and other regions linked to ichthyoses have been excluded from this family by microsatellite analysis.

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Transmission Disequilibrium of HLA-Cw*0602 and HLA-B*5701 in Psoriasis Suggests a Susceptibility Locus Between HLA-C and HLA-B

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The importance of the HLA class I alleles of the extended haplotype 57.1 (HLA-Cw* 0602-HLA-B*5701) for the genetics of psoriasis has previously been shown by several groups, including ours. To address the question whether the psoriasis susceptibility gene PSORS1 (MIM*177900) might be identical to HLA-Cw*0602 or HLA-B*5701 we studied 52 trios comprising the affected proband and both parents (156 individuals). Exon 2 and exon 3 of HLA-Cw*0602 and HLA-B*5701 were directly sequenced following haplotype specific PCR-amplification. Performing the transmission disequilibrium test (TDT) we found that only HLA-Cw*0602 and HLA-B*5701 were significantly ($p < 0.05$) associated with the disease. The TDT uses heterozygous parents to evaluate how often a certain allele has been transmitted to the affected offspring. Untransmitted alleles serve as controls. The extended TDT according to Wilson (*Ann Hum Genet* 61:151–161, 1997) gave evidence for strong association of HLA-Cw*0602 and HLA-B*5701 with psoriasis, whereas HLA-B*1301 which is known to be in linkage disequlibrium with HLA-Cw*0602 as well, showed no significant association with psoriasis. The comparison of HLA-Cw*0602 and HLA-B*5701 revealed a higher contribution of HLA-Cw*0602. However the model considering a susceptibility contribution of the risk haplotype HLA-Cw*0602-HLA-B*5701 itself led to a significantly better fit. These results indicate a susceptibility locus within the risk haplotype considered.

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Distinct Mutations in Human Basic Hair Keratins 1 and 6 Cause Monilethrix: Implications for Protein Structure and Clinical Phenotype

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Monilethrix (Mt) is an hereditary hair dystrophy recently shown to be due to mutations in the helix termination motif (HTM) of two type II human hair keratin genes, hHb1 and hHb6. It has been suggested that mutation in hHb1 produces a less severe phenotype. We have studied hair keratin genes and clinical features in 18 unrelated pedigrees of Mt from Germany, Scotland, Northern Ireland and Portugal, in 13 of which mutations have not previously been identified. By examining the rod domains of hHb1, hHb3 and hHb6, we have identified mutations in nine of the new pedigrees. We again found the glutamine-lysine substitution (E413K) in the HTM of hHb6 in two families, and in another, the corresponding E413K substitution in the hHb1 gene. In four families a similar substitution E402K was present in a nearby residue. In addition two novel mutations within the helix initiation motif of hHb6 were found in Scottish and Portuguese cases, in whom the same highly conserved arginine residue N114 was mutated to histidine (N114H) or aspartic acid (N114D) residues, respectively. In four other Mt pedigrees mutations in these domains of hHb1, hHb3 and hHb6 were not found. The mutations identified predict a variety of possible structural consequences for the keratin molecule. A comparison of clinical features and severity between cases with hHb1 and hHb6 mutations does not suggest distinct effects on phenotype, with the possible exception of nail dystrophy, commoner with hHb1 defects. Other factors are required to explain the marked variation in clinical severity within and between cases.

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Lupus Erythematosus in Patients with Type I Complement C2 Deficiency

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The gene deletion responsible for the type I human complement C2 deficiency was reported in 1992. The purpose of this study was to evaluate clinical and immunological characteristics of lupus erythematosus in patients with type I C2 deficiency.

This is a retrospective study in which all patients with a type I C2 deficiency diagnosed between 1997 and 1998 at the Laboratoire d'Immuno-Hématologie of the Strasbourg University hospital were included. In all patients, the typical 28 base pair deletion was determined by polymerase chain reaction analysis. Patient charts of 70 subjects with a proven C2 deficiency were reviewed. Ten patients had lupus erythematosus and among them, four had a complete C2 deficiency and six had a partial C2 deficiency. Seven patients had systemic lupus erythematosus, two had subacute cutaneous lupus erythematosus and one patient had chronic lupus erythematosus. Photosensitivity was present in 70% of the patients and 60% tested positive for anti-Ro(SSA) antibodies. Renal involvement, which required immunosuppressive therapy, was present in 50% of the patients. Ninety percentage of the patients tested positive for antinuclear antibodies and 60% of the patients tested positive for anti-dsDNA antibodies. Phenotyping of the fourth component of complement was performed in 80% of the patients and showed a C4A4B2 phenotype, which is suggestive for the type I C2 deficiency.

Most patients with lupus erythematosus associated to C2 type I deficiency are photosensitive and this is probably related to the presence of anti-Ro(SSA) auto-antibodies. Nevertheless, contrarily to what has been previously stated, the prognosis of those patients is not better than the prognosis of lupus erythematosus in general.

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Two Splice Site Mutations and One Deletion Mutation in the KRT1 Gene of Scandinavian Families with Epidermolytic Hyperkeratosis

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Epidermolytic hyperkeratosis (EHK) is a highly penetrant autosomal dominant genetic disorder caused by keratin gene mutations (KRT1 or KRT10). The phenotype is apparent soon after birth, induced by friction and affects most of the skin surface. Palmar-plantar regions are variably involved. Mutations cause the keratin filament network to collapse, which gives rise to epidermolysis and hyperkeratosis of the epidermis. To date, 38 different heterozygous point mutations and one deletion mutation have been identified in EHK patients (19 in KRT1 & 20 in KRT10) but no splice site mutations have been previously reported. We have now identified two splice site mutations and another deletion mutation in KRT1 from three EHK families.

All patients were examined clinically and biopsies were taken for histopathology and RNA extraction. Blood samples were collected from family members and normal controls. Both genomic DNA and mRNA were isolated and PCR (or RT-PCR) amplified portions of the KRT1 and KRT10 genes (exons 1, 6 and 7) sequenced. The mutant and normal alleles were cloned and sequenced separately and mutations were confirmed by RFLP analysis.

Two patients had a heterozygous point mutation of the exon-intron 1 splice donor site (GT to CT in one and GT to AT in the other) of KRT1. This caused the mRNA product to be shortened in one case (GT/CT) and lengthened in the other (GT/AT) due to aberrant splicing. Both alterations led to the loss of an Hph I site producing a diagnostic fragment of 199 bp. The third patient had a heterozygous deletion mutation (12 bp fragment of KRT1 exon 1). This encoded part of the H1 domain and resulted in the removal of four amino acid residues (EIDP), significantly shortening the H1 domain. A Dde I site was lost giving a diagnostic fragment of 744 bp. These alterations are potentially disruptive to protein structure and the resulting keratin filament instability is sufficient to explain the observed pathology.

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Three Novel Heterozygous Point Mutations in Keratin Genes (KRT1 and KRT10) in Families with Epidermolytic Hyperkeratosis

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Epidermolytic hyperkeratosis (EHK) is an autosomal dominant genetic disease that results from mutations in the KRT1 or KRT10 genes. The phenotype is highly penetrant and affects most of the skin surface. It is induced by friction, which causes collapse of the keratin intermediate filament system, altered gene expression and a cellular reaction typified by epidermolysis and hyperkeratosis. To date 38 different heterozygous point mutations have been identified in EHK patients (18 in KRT1 and 20 in KRT10). We have now examined three Scandinavian families and found three new heterozygous point mutations, two in KRT1 and one in KRT10. All three index cases showed palmar-plantar involvement.

Scandinavian patients with EHK were examined clinically and blood samples collected from family members and normal controls. Genomic DNA was isolated and both exons 1 and 7 of KRT1 and KRT10 were amplified by PCR with specific primers. The fragments were sequenced (manual and automated) and the mutations confirmed by restriction enzyme digestion of the PCR products and gel analysis (1.5% Agarose or 4% NuSieve Agarose).

One patient had a point mutation (AAG to AAT) in exon 1 of KRT1. This encoded the H1 domain and altered codon 177 from lysine to asparagine (K177N). This created an Eco RI site so the mutant allele was cleaved into two diagnostic fragments (610 bp and 214 bp). The second mutation was TTT to TTG also in exon 1 of KRT1 altering residue 11 of the 1 A helix from phenylalanine to cysteine (F191C). Finally, we identified a new mutation (CTA to CCA) in exon 6 of the KRT10 gene. This altered residue 114 of the 2B helix from leucine to proline (L453P) and resulted in loss of a Bfa I site from the mutant allele (diagnostic fragment of 399 bp). These alterations are all potentially disruptive to protein structure and the resulting keratin filament instability is sufficient to explain the observed trauma induced pathology.

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CDKN2A Novel Mutation in a Patient from a Melanoma-Prone Family

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About 10% of melanoma is inherited with an autosomal dominant model with variable penetrance. A locus for familial melanoma has been mapped in 9p21 and, the CDKN2A (p16, $p16^{ink4a}$, MTS1) tumor suppressor gene, is considered as the main candidate gene for melanoma susceptibility. Deletions or mutations in the CDKN2A gene may produce an imbalance between functional p16 and cyclin D causing an abnormal cell growth. In fact, the normal p16 protein binds to CDK4 that became unable to interact with cyclin D, controlling the passage through the G1 checkpoint of the cell cycle.

We describe a novel mutation consisting in a 1-bp deletion at codon 67 that produce a frameshift and a 145 stop codon. The analysis was performed by using PCR-heteroduplex analysis and direct DNA sequencing.

As already stressed by some authors a mutation in the conserved areas of the ankirin repeats (spanning amino acids 11–142), is capable to disrupt the CDKN2 protein primary strupture producing a loss of the protein inhibitory function.

The patient, a female subject from a melanoma prone-family, showed, at the age of 47, a superficial spreading melanoma onset on the anterior trunk, 1.20 mm Breslow index, IV Clark level, with a moderate spontaneous regression. The father was affected either by melanoma and colon cancer, the uncle by gastric cancer and the grandfather by larynx cancer. We discuss the significance of this mutation in the susceptibility to melanoma and other tumors.

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Frequency of the Interleukine-4 Receptor Mutation Q576R in an Atopic Cohort

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Atopic disease, such as atopic dermatitis and asthma, is a genetic predisposition to develop allergies that affect up to 20% of European population. Interleukine-4 and interleukine-4 receptor genes are associated with atopy. Interleukine-4 enhances the TH2 lymphocytes and improves immunoglobuline isotype switching into epsilon.

In a series of 20 atopic patients, Hershey has reported a 20% rate of Q576R mutation in the interleukine-4 receptor compared with 10% in 30 controlled patients ($p = 0.001$). He first described this mutation in three Job's syndrome patients. This results was not confirmed by Grimbacher studies carried out on a population of 20 Job's syndrome patients (5% and 12% mutation's rate). The Q576R mutation impairs the signalling pathway of the receptor by reducing the fixation at 575 of SHP-1, a phosphotyrosine phosphatase involved in the termination signal. So the receptor function is increased. Thus, evaluating mutation frequency among a large atopic cohort to assess its responsibility would be worthwhile.

We selected 100 unrelated atopic patients followed in Dermatology and Pneumology Departments for atopic dermatitis or asthma. Patients and staff without atopic diseases constituted a 200-individual control group. DNA was extracted from circulating lymphocytes. Amplification was performed with a specific 5'primer creating an Ava-1's restriction site in the mutant allele. After digestion, migration in a 2% agarose gel segregated wild type and mutant alleles. Up to now a 17% rate of mutant chromosomes was found among 64 atopic patients, and a 15% one among 128 controlled patients; these results are not statistically different ($p = 0.78$). This study indicates thus that atopic disease is not induced by interleukine-4 receptor Q576R mutation.

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Structural Basis for the Impaired Barrier Function in Netherton's Syndrome: Differentiation from other Infantile Erythrodermas

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The infant with Netherton's syndrome (NS) typically displays a erythroderma, which can be difficult to distinguish from erythrodermic psoriasis (PsoE), nonbullous congenital ichthyosiform erythroderma (CIE), or other infantile erythrodermas. Some NS infants develop progressive hypernatremic dehydration, a complication which can be fatal. The diagnosis typically is delayed until the appearance of the pathognomonic trichorrhexis invaginata. To facilitate the early diagnosis of NS, biopsies from seven patients with erythrodermic NS were compared with that of three patients with PsoE, and two patients with CIE. They were processed for light and electron microscopy using postfixation with both OsO4 and ruthenium tetroxide. A distinctive feature, premature secretion of lamellar body (LB) contents, occurred only in NS. LB-derived extracellular lamellae and SC lipid membranes were separated extensively by foci of electron-dense material. Transformation of LB-derived lamellae into mature lamellar membrane structures was disturbed in NS. Premature LB secretion and foci of electron-dense material in the intercellular spaces of SC, features that were not observed in the other erythrodermic disorders, appear to be frequent and relatively specific markers for NS. These ultrastructural features could permit the early diagnosis of NS, prior to the appearance of the hair shaft abnormally. The abnormalities could explain the impaired permeability barrier in NS, accounting for the hypernatremia and dehydration in infants with NS.

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Lack of Suspected A142R, A141R TGK Mutations in Hungarian Patients with Autosomal Recessive Ichthyosis

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Autosomal recessive ichthyosis (ARCI) is a genetically heterogenous group of disorders. Keratinocyte transglutaminase (TGase I) mutations have been reported in both clinical subgroups of the disease, lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE). Five patients with CIE and two patients with LI, from seven unrelated families have been investigated. The diagnosis was confirmed by histological and ultrastructural analysis. Based upon repeatedly observed mutations of the TGase I gene at the 141 and 142 aminoacid positions in Finnish ARCI patients, we analysed DNA of our patients using PCR and restriction enzyme technique (HhaI and Sac II) to identify possible mutations in this region. The lack of suspected A141R and A142R mutations correlated with the results of TGase I activity assays using the monodansylcadaverine-antidansylcadaverine immunofluorescent method, indicating TGase activities at the distribution corresponding to TGase I.

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Genetic Mosaicism in an Acquired Inflammatory Dermatosis Following the Lines of Blaschko

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There is increasing evidence that genetic mosaicism plays a crucial role in many hereditary and tumoral disorders. In this report, we were able to show a genetic mosaicism in a patient with an acquired inflammatory dermatosis along the lines of Blaschko.

A 38-y-old woman developed an extensive, Blaschko-linear, erythematosquamous dermatitis involving the face, the four limbs and the trunk. A cutaneous biopsy showed a lymphocytic dermal infiltrate and mild spongiosis. The lesions regressed spontaneously within 4 wk. We performed cutaneous biopsies within diseased skin and at adjacent normal appearing skin for cell culture and chromosome analysis on dermal fibroblasts using standard methods. Cytogenetic studies revealed a normal 46, XX karyotype on 98 mitosis in normal appearing skin, while an abnormality including a chromosome 18 was found in five out of 100 mitosis in diseased skin. Chromosome analysis of peripheral blood lymphocytes showed a normal 46, XX karyotype.

For the first time, we were able to demonstrate a genetic mosaicism in an acquired Blaschko-linear inflammatory dermatosis. The mechanisms through which genetic mosaicism can lead to such a linear inflammatory dermatosis remain yet hypothetical. This genetic mosaicism could be responsible for cutaneous antigenic mosaicism, the expression of which might be induced by a viral infection for example. This viral infection could then trigger an inflammatory T-cell response in a Blaschko-linear fashion. This is the first report showing that genetic mosaicism can also play a role in inflammatory diseases. The demonstration of genetic mosaicism in an acquired inflammatory Blaschko-linear dermatosis strongly supports the clonal hypothesis of Blaschko lines.

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Cutaneous Phenotypes Associated with Susceptibility to Internal Malignancies?

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Characteristic skin lesions can be found in the context of family cancer syndromes such as Gorlin's, Muir-Torre and Cowden's syndromes. In atypical mole syndrome (AMS) multiple naevi are associated with increased susceptibility to melanoma. The aim of this study was to assess the cutaneous phenotype of patients from families prone to internal malignancies with or without skin cancers. A total of 130 individuals with familial clustering of internal neoplasms underwent a detailed skin examination. Fifty-three individuals were from 19 families in which between two and seven family members were examined. In 26 families the AMS was found in the context of familial susceptibility to a variety of non cutaneous malignancies with or without melanoma. In contrast to previous work (Bergman W, Watson P, de Jong J *et al*: Systemic cancer and the FAMMM syndrome. *Br J Cancer* 61:932–936, 1990) there was no obvious preponderance of gastrointestinal tumours in the AMS group. Another phenotype encountered in 11 other cancer prone families was characterised by the presence of multiple macular pigmented lesions resembling flat seborrheic keratoses on sun exposed areas of the forearms and/or lower legs. Histology was reviewed on 24 of these lesions from 10 individuals (mean age 56 y, range 43–69 y) belonging to nine families, revealing features in keeping with stucco keratosis (eight lesions), flat seborrhoeic wart (11), lentigo (three) and squamous cell papillomata (two). No epidermal dysplasia was noted. Six of these nine families were characterised by bowel cancer susceptibility.

The AMS appears to be a marker of susceptibility not only to melanoma but also to internal malignancies. The presence of multiple stucco keratoses and macular seborrhoeic warts in cancer prone families suggests that this phenotype may also have potential as a marker of cancer susceptibility especially in bowel cancer families.

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A Case of Epidermolysis Bullosa Simplex (Köebner): Genetic and Ultrastructural Study

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A female preterm baby, 31 wk 2 d, 1570 g, was suffered from bullae and erosion on her buttock and extremities since day two of her birth, without family history of epidermolysis bullosa. Skeletal abnormality was not observed, and the skin lesion was gradually epithelized without leaving scar or milia formation. Skin biopsy revealed intraepidermal bullae in basal layer of the epidermis. Cytolytic change in the basal cells was confirmed on electron microscopic examination. Mutation analysis of cytokeratin 5/14 gene was performed.

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Cutaneous Manifestations of Familial Mediterranean Fever in Republic of Georgia

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Familial Mediterranean Fever (FMF) is generally uncommon, but in endemic regions well known disease. Mainly patients are Jewish, Armenian or Turkish origin. Small numbers of cases are described in other ethnic groups too. One of them is Georgians.

Skin manifestations are common sign of FMF. According to literature, frequency of dermatological signs differs in different ethnic groups.

We studied frequency of dermatological manifestations of patients with FMF registered in I Clinic of Medical University Tbilisi, Georgia (specialized Center of FMF research).

Thirteen patients with FMF, citizens of Tbilisi were registered. Original Questionnaire was completed in all 13 cases. Six patients (46%), all of them ethnic Georgians have had erysipelas-like erythema. In all cases rush appears before (max. 6 h) attack of arthralgia and is present mainly 72 h. Rush solves simultaneously with arthralgia.

Frequency of dermatological manifestations is equal with frequency of attacks of arthralgia. Prophylactic treatment of FMF with colchicum reduces frequency of arthralgick attacks and proportionally frequency of rush. The character of skin manifestation stays unchanged.

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Hepatocyte Growth Factor/Scatter Factor Increases Cutaneous Wound Healing in the *Diabetes* Mouse

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Hepatocyte growth factor (HGF/SF) is a heparin binding polypeptide that stimulates cell proliferation, motility and morphogenesis via activation of its tyrosine kinase receptor c-Met. HGF/SF has also been identified as a potent angiogenic factor in a sponge implant model in rats. To evaluate the potential of HGF/SF to heal cutaneous wounds we have topically applied recombinant murine HGF/SF to full thickness excisional injuries made on the *diabetes* mouse. The *diabetes* mouse is the result of a spontaneous mutation in an inbred mouse strain. Homozygote animals (*db/db*) are obese, hyperglycemic and exhibit impaired cutaneous wound healing compared to their wildtype littermates.

Full thickness circular wounds (6 mm diameter) were made on either shaved dorsolateral flank of 8–9-wk-old animals to provide treatment and control sites. The wounds were covered using an occlusive dressing and HGF/SF or PBS was administered through the dressing daily for up to 6 d post wounding. In one set of experiments the animals were killed on day 10, the wounded tissue was excised, wax embedded and H & E stained sections were used to assess the level of wound healing. Alternatively, on day 10 a similarly treated group of animals were injected i.v. with 1 ml of 25% carmine dye/10% gelatin solution. Following cooling, the wound and surrounding tissue was excised, and the dye content assessed spectrophotmetrically to provide blood vessel volume within the tissue.

Initial macroscopic assessment following tissue removal indicated that healing of wounds treated with HGF/SF was more advanced than wounds treated with PBS alone. This was confirmed when measurements demonstrated that HGF/SF treatment significantly ($p < 0.05$) increased granulation tissue thickness and re-epithelialisation compared to PBS treated wounds. HGF/SF treated sites had a blood vessel volume of 5.8 ± 1.2 nl per mg compared to 4.5 ± 1.5 nl per mg in PBS treated sites indicating significantly ($p < 0.05$) more angiogenic activity in HGF/SF treated wounds.

In summary, HGF/SF increases healing of impaired full thickness cutaneous wounds by increasing granulation tissue which provides a framework for keratinocytes to migrate across the wound, thus, aiding the process of re-epithelialisation. Moreover, HGF/SF has the potential to enhance the mitogenic and motogenic properties of this cell population further augmenting wound re-epithelialisation. The granulation tissue may also be acting as a matrix allowing the localisation of greater numbers of leukocytes within the wound site, an attribute that will be assisted by the elevated blood flow seen in HGF/SF treated wounds.

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RTH16 is a Potent Modulator of VEGF Expression (Protein and Gene) in Human Hair Cultured Dermal Papilla Cells *In Vitro*

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The growth factors susceptible to promote angiogenesis and vasodilation are the best candidates for inducing and maintaining hair bulb vascularization because the telogen stage is associated with a disappearance of vascular network in hair dermal papilla.

We were particularly interested on VEGF (Vascular Endothelial Growth Factor): a potent angiogenic and vasodilator factor which is mitogenic for endothelial cells *in vitro*. Hair dermal papilla present the receptors of this factor and produce it *in vitro* (Lachgar *et al*: *J Invest Dermatol* 107:17–23, 1996).

A hair cycle-dependent change in the expression of VEGF protein and mRNA is observed with a strong expression of this factor by dermal papilla cells at the anagen stage (Lachgar *et al.*, In *Hair for the Next Millenium*. Eds D.J.J. Van Neste, V.A. Randall Amsterdam: Elsevier, 1996, pp. 407–412).

This study was initiated to evaluate the *in vitro* angiogenic activity of a breveted vegetal extract RTH16 (ANASTIM, DUCRAY) approved recently for the prevention of hair loss on two steps of VEGF regulation (protein and gene) in human cultured hair dermal papilla cells.

A comparative study was performed in parallel with a vasodilator molecule which induces an upregulation of VEGF (Lachgar *et al.*: *Br J Dermatol* 138:407–411, 1998).

Our results concerning VEGF protein assay after 24 h of cell treatment show that the RTH16 strongly stimulates dose-dependently VEGF production in both supernatants and cell extracts.

The time-course of VEGF production in the presence of one concentration of RTH16 (100 μg per ml) presents a high activity of RTH16 within 24 h (+110%).

RT-PCR analysis demonstrates a positive regulation of VEGF gene: early significant stimulation of VEGF expression in dermal papilla cells is observed after 3 h of treatment with 100 μg per ml of RTH1 6. In conclusion, these results demonstrate the presence of a strong positive regulation at transcriptional level. The early stimulation obtained with RTH16 on VEGF, a determinant factor of vascular cell responses, reflects the significant fast angiogenic potentiality of this extract in hair follicle dermal papilla cells. Therefore these data suggest its implication in hair dermal papilla vascularization, an important parameter for maintaining hair follicle in the anagen stage.

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Signal Characteristics of EGF-Induced Protein Tyrosin Phosphorylation in Psoriatic Keratinocytes

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Although tyrosin phosphorylation is an early event required for cell proliferation as well as for cell differentiation and a higher activity of protein tyrosine kinases (PM) is known in psoriatic keratinocytes (M), the PTK dependent phosphorylation of proteins lying downstream of the EGF-receptor family has yet not been observed in psoriatic KCs. In addition to the well known receptor PTK-activity induced by the EGF-R PTK family, time-dependent tyrosine phosphorylation of a few unidentified proteins and of members of the SRC-family (particularly src and fyn) in keratinocytes cultivated from men and from rats were described recently.

To study in intracellular phenomenon of tyrosine phosphorylation in KC, particularly in psoriatic KC, first we determined the time-dependency (5–30 min) of protein tyrosine phosphorylation using EGF-stimlated HaCaT and psoriatic KC. Second, cultured KC from psoriatics ($n = 8$) or controls ($n = 11$) were treated with EGF (10 ng per ml) over 15 min. In some experiments, PKC was additionally blocked with Ro 31–8220. Cells were lysed in buffer, cellular protein was immunoblotted after SDS page separation with mABs against either anti-phosphotyrosin (PY20) or anti-p60sre. The pattern of tyrosin phosphorylated protein bands was strongly different in the fracton derived from psoriatic KC compared to KC from controls. The most interest result was the highly reduced staining of p60 src in psoriatic keratinocytes. SRC phosphorylation and elevated src-activity is known in calcium and vitamin D dependent signaling in human KC. In contrast to those calcium or vitamin D dependent mechanisms which are known to induce KC differentiation, an absence or inhibition of p60 src in psoriatic KC could be responsible for the elevated proliferation rate of psoriatic KC and could act therefore as a key mechanism in the cell cycle control of psoriatic KC.

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Rho-kinase, cAMP and cGMP Interfere with the Maintenance of Contractile Forces Generated by Fibroblast Populated Collagen Lattices

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The aim of this investigation was to study the effect of Rho-kinase inhibition, cAMP and cGMP on the maintenance of contractile forces generated by fibroblast populated collagen lattices (FPCL). The isometric force generated by 3-dimensional FPCLs of 0.8 ml volume (cross-sectional area: 63 mm^2), with a collagen concentration of 1.75 mg per ml and a cell concentration of 62500 per ml was measured using a previously described procedure (Pflügers Arch. Suppl. to Vol. 435 R170, 1998). After stimulation with fetal calf serum (FCS, 10%) a plateau force (PF) of 578 ± 19 μN ($n = 30$) was generated and 3.1 ± 0.07 h ($t_{\frac{1}{2}}$) were required to reach half the PF. Addition of Y-27632, a specific Rho-kinase inhibitor, relaxed the PF dose dependently ($IC_{50} = 0.67$ μM, $n = 8$–12). A concentration of 10 μM Y-27632 caused a relaxation ($t_{\frac{1}{2}}$ 14 min) which could not be further reduced by 2 μM cytochalasin D. In contrast forskolin (400 μM) or dibutyryl cAMP (1 mM) with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) under the same conditions induced only a partial relaxation by $61 \pm 4\%$ ($n = 5$, $t_{\frac{1}{2}}$ 44.6 ± 3.9 min) and $64 \pm 7\%$ ($n = 4$, $t_{\frac{1}{2}}$ 74.25 ± 4.3 min), respectively, with a complete force reduction by subsequent addition of 2 μM cytochalasin D. Equally the addition of the NO donors sodium nitroprusside (SNP, 5 mM) and S-nitroso-N-acetyl-penicillamine (SNAP, 0.5 mM) only resulted in an incomplete relaxation. SNP caused a biphasic relaxation (1.: $10.6 \pm 2\%$, $t_{\frac{1}{2}}$ 20.4 ± 2.8 min; 2.: $51.4 \pm 5{,}7\%$, $t_{\frac{1}{2}}$ 4.6 ± 0.67 h; $n = 4$) whereas SNAP induced a monophasic reduction of PF ($27 \pm 5\%$, $t_{\frac{1}{2}}$ 37.2 ± 2.4 min; $n = 3$). 8-pCPT-cGMP, a membrane permeable cGMP analogue, caused a monophasic relaxation by $45 \pm 4\%$ ($t_{\frac{1}{2}}$ 53.76 ± 2.5 min; $n = 3$). The PF was unaffected by the addition of the Ca^{2+} chelator BAPTA-2AM at a concentration of 0.01–1 mM ($n = 3$). These results demonstrate that the isometric force generated by FPCLs is relaxed completely by inhibition of Rho-associated kinase but not by cAMP or cGMP. This supports the hypothesis that Rho-kinase positively and cAMP and cGMP negatively regulate the force maintenance by human dermal fibroblasts within a collagen matrix.

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UVB Irradiation Mimics Ligand-Induced Activation of Keratinocyte Growth Factor Receptor

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UV irradiation of mammalian cells induces activation of growth factor receptors, such as Epidermal Growth Factor receptor, and triggering of signal transduction pathway. This UV-response is mediated by the production of reactive oxygen species (ROS), which results in oxidative stress, that can be blocked by antioxidants. We analysed the effect of physiological doses of UVB and that of the pro-oxidant agent cumene hydroperoxide (CUH) on the receptor for Keratinocyte Growth Factor (KGF), a key mediator in epithelial growth and differentiation. The KGF receptor (KGFR) is a receptor tyrosine kinase, splicing variant of the fibroblast growth factor receptor 2, expressed only on cells of epithelial origin. Exposure to both UVB (300 mJ per cm^2) and CUH (200 μM) of NIH 3T3/KGFR transfectants caused tyrosine phosphorylation of KGFR, as observed after 10 min, similar to that induced by KGF. N acetyl cysteine inhibited UVB-induced receptor phosphorylation. The KGFR expression appeared unmodified by the treatments. Ultrastructural observations of both UVB and CUH treated cells showed a normal morphology of the plasma membranes and intracellular organelles. A decrease of polyunsaturated fatty acids of cell membranes was observed after both treatments associated with a decrease of catalase activity and GSH level, whereas superoxide dismutase activities were unchanged. Similar effects were observed on NIH 3T3 untransfected control cells. Taken together our results demonstrate that exposure to pro-oxidant stimuli induces a rapid intracellular production of ROS, which are in turn capable of triggering KGFR activation

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The Effects of Sphingolipid Derivatives and Synthetic Pseudo-Sphingolipids on Epidermal Proliferation and Differentiation

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Eczematous dermatitides is a group of skin diseases characterized by inflammatory hyperproliferaton and abnormal differentiation of epidermis. The most common types are atopic dermatitis of genetic predisposition and allergic or irritant contact dermatitis by environment injury. PKC plays a key role as a mediator of inflammation and growth in epidermal keratinocyte. Therefore, PKC inhibitors such as D-sphingosine are regarded as target substances in the development of anti-inflammatory skin disease agents. We identified several kinds of inhibitors from sphingolipid derivatives and synthetic pseudosphingolipids, that can inhibit PKC activity *in vitro*. We tested these compounds for their ability to inhibit in an assay system using the repeated-barrier disruption model or TPA-induced hyperplasia/inflammation model of hairless mice skin. These compounds inhibited the epidermal hyperplasia and inflammation induced by repeated epidermal injury or phorbol ester. Moreover, these compounds also enhanced the activity of transglutaminase, a late differentiation marker and accelerated the formation of cornified envelop in primary human epidermal keratinocytes. The results therefore suggest that the inhibitors are putative potent regulator of proliferation and differentiation of epidermal keratinocytes. These compounds could be developed into useful therapeutic agent of eczematous dermatitides such as atopic dermatitis, allergic or irritant contact dermatitis.

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Melanoma-Associated Antigen Expression in Primary Melanoma: Frequency of Antigen Loss and Correlation with Clinical Outcome

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Melanoma-associated antigens are cornerstones in the context of many immunotherapeutic trials aiming to elicit an antimelanoma immune response. However, little is known about expression of these antigens in primary melanoma and the correlation with the natural course of disease.

We randomly selected 110 patients with known follow-up for at least 10 y from our melanoma unit. Primary melanoma specimens which were formalin-fixed and paraffin-embedded were stained for MAGE-3, MelanA/MART-1, gp100, tyrosinase and HLA class I using monoclonal antibodies 57B, A103, HMB45, T311 and vf135–1D6, respectively. Staining was rated on arbitrary scales for intensity and distribution pattern. Primary end-point for clinical outcome was death. Data was analysed by Kaplan-Meier method using logrank test and Cox regression with forward log ratio.

Ninety-one patients qualified for evaluation (age range 22–89, median age 59.9; men 58%, women 42%). Antigen loss for MelanA was found in 58% of primary tumors, for MAGE-3 in 27%, for gp100 in 72% and for tyrosinase in 59%. HLA class I showed a loss in 73% of primary melanomas. Breslow thickness was indicative of cumulative survival ($p = 0.0384$). However, no significant statistical correlation could be found between loss of antigens MAGE-3, MelanA, gp100, or tyrosinase and cumulative survival ($p = 0.07$ or higher); or HLA class I status, antigen expression and cumulative survival; or between antigen expression, Breslow thickness and cumulative survival.

In our study of 91 patients with primary melanoma, only Breslow thickness significantly correlated with survival. Melanoma-associated antigens or HLA status showed no statistical significance. Loss of melanoma-associated antigen expression alone is not a useful parameter for prognosis in primary melanoma.

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Variants of Melanocortin 1 Receptor (MC1R) Gene and Non-Melanoma Skin Cancer

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Variants of the melanocortin 1 receptor (MC1R) gene are associated with fair skin and red hair. Conflicting results have been reported for the association between this MC1R gene polymorphism and cutaneous melanoma. It is not known whether subjects carrying 1 or 2 variant alleles of the MC1R gene have an increased risk for the development of nonmelanoma skin cancer, which question was adressed in this study.

In a case control study, the MC1R alleles were determined in 72 subjects with cutaneous squamous cell carcinoma (SCC), in 236 subjects with basal cell carcinoma (BCC) and in 255 controls. SSCP analysis and direct sequencing were used for determining wild type and variant alleles. For each subject skin type was assessed. Crude odds ratios and odds ratios adjusted for skin type were calculated and interpreted as relative risks for the development of nonmelanoma skin cancer.

The crude odds ratios for developing SCC in persons with one or two variants of MC1R, respectively, compared with no variant were 1.24 (95%CI 0.60; 2.59) and 2.75 (95%CI 1.26; 6.05). After analyses, stratified for skin type, the adjusted odds ratios (Mantel-Haenszel) were 1.14 (95%CI 0.54; 2.46) and 2.10 (95%CI 0.94; 5.06), respectively. The crude odds ratios for developing BCC in persons with one or two variants of MC1R, respectively, compared with no variant were 1.26 (95%CI 0.80; 1.98) and 1.92 (95%CI 1.13; 3.27). After analyses, stratified for skin type, the adjusted odds ratios (Mantel-Haenszel) were 1.22 (95%CI 0.76; 1.95) and 1.50 (95%CI 0.82; 2.72), respectively.

The presence of two variant alleles of the MC1R gene is likely to be associated with the development of both SCC and BCC. The association of MC1R variants with SCC appears to be stronger than with BCC. Skin type was shown to be a slightly confounding factor in the association between two variants of MC1R and the development of both SCC and BCC. Additional studies are needed to confirm the association between MC1R gene variants and nonmelanoma skin cancer.

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Increase in Basal Cell Carcinomas Localized on the Trunk. A 30 y Study

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A rise in basal cell carcinoma (BCC) incidence has been observed in many Western countries during the past 10–20 y. It has been suggested that a common epidemiological factor could be involved in both superficial BCC and thin melanomas. We retrospectively studied the demographic data, localization and histologic variants of all BCC diagnosed in our laboratory of dermatopathology during the period 1967–96.

All cases of BCC confirmed by two dermatopathologists were included in this study. A total of 13478 cases were recorded, occurring in 12261 patients. The median age was 65 years. The CBC were localized on the face & neck in 81.7% of cases, on the trunk in 11.5% and on the limbs in 5.1%. The proportion of BCC of the trunk regularly increased by +0.18% per year (from 11.5% in 1967 to 14.7% in 1996), whereras we noticed a regular decrease of –0.35%/year in the proportion of BCC of the head & neck. This rise in BCC of the trunk was due to both superficial and nodular forms of BCC, but mainly to the nodular BCC which increased by +0.40%/year. We have not found a significant rise in the BCC occurring in patients under 40 y of age. The comparison of the superficial BCC with superficial spreading melanomas (independanlty of their localization) during the period 1980–96 showed a similar linear increase in incidence of both tumors.

Our study shows a global increase in the number of BCC analyzed each year, but this could be due in part to inclusion bias (retrospective study, increase in the number of dermatologists in our area, increase in elderly patients during the 30 last years). The rise in BCC of the trunk observed in this study contrasts with the regular increase in mean age of our patients with BCC (from 62.9 to 67.2 y), since BCC of the trunk are observed in younger patients. Although this a retrospective study based on dermatopathological files, this almost linear increase in the proportion of BCC of the trunk is likely to be significant. It could be related to changes in the sun exposure habits during the past 30 y. The similar increase in superficial BCC and thin melanomas in the same geographical area could also be interpreted as a common epidemiological factor, but this remains to be established.

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Reactivity of Classical Kaposi's Sarcoma and Blood Donor Sera with a Major Defined Epitope of HHV-8 Small Viral Capsid Antigen

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Serum samples of classical Kaposi's sarcoma patients ($n = 12$) and Hungarian blood donors ($n = 120$) were tested with peptide ELISA and anti-LANA (HHV-8 latent nuclear antigen) immunofluorescence. In the ELISA a 14 aa synthetic oligopeptide from the carboxyterminus of orf65/minor capsid protein (aa 157–170, AVADARKPPSGKKK) was used as antigen. This has been identified as a major defined epitope of human herpesvirus-8 (HHV-8) small viral capsid antigen (sVCA). Six of 12 sera obtained from patients with classical KS were reactive in ELISA whereas all had antibodies to LANA. Titers of anti-LANA antibodies in KS patients were high (640–5120 (mean = 3495). In the control blood donor group two of the 120 had antibodies to LANA with low titers (<160) and 15 of the 120 (12,5%) healthy blood donors had antibodies to the sVCA derived oligopeptide antigen. The different prevalence (Fisher-exact-test, $p < 0.01$) and titer (Mann–Whitney $p < 0.001$) of the anti-LANA antibodies were significant between the two populations. The prevalence of HHV-8 infection in the general Hungarian population seems to be higher than our previous anti-LANA studies suggested. Since the high titer reactivity of KS patients sera with LANA were more consistent and specific to KS than reactivity to sVCA we suggest its usefulness in differential diagnosis. Detection of antibodies to sVCA could be more important inepidemiological studies to assess the prevalence of HHV-8 infection in the general population than as a diagnostic tool.

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Skin Cancer in Heart Transplant Recipients: Analysis of Risk Factors and Relevance of the Immunosuppressive Therapy

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The frequency of skin cancer is increased among organ transplant recipients, but the predisposing risk factors are controversial. In particular, there are some data indicating that the increased risk of skin cancer may be related more to the overall immunosuppressive load than to the type of immunosuppressive regimen. However, it is difficult to accurately evaluate immunosuppressive load in a large group of patients. We have therefore considered the 1st year postheart transplant (HTx) rejection score (1) as an indirect marker of the level of immunosuppression. Patients with a higher 1st year post-HTx rejection score received a higher immunosuppression.

We performed multivariate analysis of risk factors for skin cancer in 272 heart transplants (aged 49 ± 14 y, 233 male, mean follow-up 4,7 y, range 1 mo–12 y) followed up at a single Center. An extensive dermatological examination was carried out; baseline features, type of immunosuppressive regimen, 1st year post-HTx rejection score, extent of sunlight exposure, skin type were recorded. Multivariate analysis (Cox regression) included: age at transplantation, sex, skin type, type of immunosuppressive regimen, 1st year post-HTx rejection score, presence of warts, sunlight exposure.

The cumulative incidence of skin cancer by life table analysis increased from 15% after 5 y to 34% after 10 y. By multivariate analysis, age at transplant >45 y ($p = 0.03$; $R = 3$), skin type 2 ($p = 0.005$; $RR = 3$), sunlight exposure >1.00.00 h ($p = 0.01$; $RR = 2.5$), and 1st year post-HTx rejection score > 18 ($p = 0,05$, $RR = 2$), but not type of immunosuppressive regimen were significant risk factors.

Our study confirms that age at transplant, skin type and sunlight exposure are relevant risk factors for skin cancer in transplant recipients. Moreover, our investigation suggests that the degree of immunosuppression may represent a more important risk factor than the type of immunosuppressive regimen.

(1) Billingham ME, Cary NRB, Hammond Me *et al*: A working formulation for the standardisation of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. *J Heart Lung Transplant* 9:587–593, 1990

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Analysis of Cytokine Expression in Cutaneous Warts During Imiquimod Treatment by Microarray cDNA Hybridization

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The immune response modifier imiquimod has been shown to be effective in the treatment of HPV 6/11-containing genital warts. It is suggested that its antiviral action results from *in vivo* cytokine activation of the immune system. Pilot studies point out that imiquimod has also an effect on cutaneous warts which show much stronger keratinization than genital lesions. Therefore, we were interested to find out if local application of imiquimod might cause detectable changes in cytokine expression of cutaneous warts as well. mRNA was extracted from three untreated HPV 2/57-induced cutaneous warts and from three HPV 2/57 warts during local treatment with 5% imiquimod cream twice daily. After reverse transcription, cDNA was amplified by degenerate oligonucleotide primed PCR. Differential gene expression was demonstrated by SSCP gel electrophoresis of PCR products and by hybridization of labelled PCR products on a microarray membrane yielding 268 different cDNAs of cytokines and cytokine receptors. SSCP gel analysis revealed significant and reproducible changes in gene expression during imiquimod treatment. Microarray hybridization showed upregulation of B-cell growth factor, MRP-8 and G-CSF.

Our results suggest that imiquimod application has an effect on cytokine expression in cutaneous warts which may explain the clinically observed efficacy of imiquimod in the treatment of cutaneous warts.

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Integrin α6 is not the Obligatory Cell Receptor for Mucosotropic Papillomaviruses

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Recently integrin α6 has been proposed as the epithelial cell receptor for papillomavirus (Evander *et al*: *J Virol* 71:2449–2456, 1997). We have investigated whether α6 integrin is the cellular receptor for bovine papillomavirus type 4 (BPV-4) which is strictly epitheliotropic and infects the mucous epithelium of the upper digestive tract. Primary bovine mucosal keratinocytes from the palate of a fetus (PA1K) display high levels of α6 integrin; matched primary fibroblasts from the same biopsy (PaIF) express almost no α6 integrin. BPV-4 however, binds both PalK and PalF to similar saturable levels. Native BPV-4 virions infect PalK *in vitro* as detected by RT PCR of E7 mRNA. Infection can be blocked by neutralising antisera against LI-L2 and LI VLPs, or by denaturation of the virions, supporting the view that *in vitro* infection mimics the *in vivo* process. Human keratinocyte cell lines were derived from patients with genetic lesions in their hemidesmosomes. The level of α6 integrin expression was determined in these cell lines by *in situ* immunofluorescence and FACS analysis and compared to primary keratinocytes. Wild type keratinocytes KHSV and α6 integrin-negative keratinocytes KHA and KHBOUA were all infected by BPV-4 apparently to the same extent of PalKs. These results are consistent with α6 integrin not being the obligatory receptor for this mucosotropic papillomavirus.

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Expression of HIV CXCR4, CCR5 and CD4 Receptors on Fresh and Cultured Human Langerhans Cells and Effects of Cytokines

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Langerhans cells are located in epidermis and some mucosae. When stimulated by foreign antigens, they migrate to lymph nodes and become interdigitating cells resembling cultured Langerhans cells. Langerhans cells are probably the first infected cells by HIV. They are known to express CD4 and CCR5 molecules, HIV receptors. The expression of CXCR4, HIV receptors for X4-tropic variants, has been shown in the cytoplasm but not on cell membrane. The aims of this study were to detect CXCR4 expression on fresh Langerhans cell membrane and to follow CXCR4, CCR5 and CD4 expression on Langerhans cell surface, under stimulation by different cytokines. Langerhans cells were isolated from skin and highly purified before culture. Using flow cytometer, CXCR4 was found on fresh Langerhans cell membrane. The percentage of Langerhans cells expressing CXCR4 or CCR5 increased in culture in usual conditions. The number of CD4, CXCR4 and CCR5 molecules did not significantly change. Moreover, we investigated evolution of coreceptor expression with cytokines. GM-CSF strongly inhibited the percentage of CCR5+ cells but enhanced the number of CXCR4+ cells. In a similar manner, TNFα diminished the percentage of CCR5+ cells without modifying CXCR4 expression. IL-10 induced an increase of the density of CCR5 molecules at low concentration and a decrease of the density of CCR5 molecules at higher concentration. In this study, we have demonstrated CXCR4 expression on Langerhans cell membrane. The expression of CXCR4 and CCR5 on Langerhans cell membrane strongly suggests that these cells could be infected by both X4 and R5 viruses.

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The Effect of Viral Interferon Regulatory Factor of Human Herpesvirus 8 in Human Epithelial Cells Following Interferon Treatment

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Human herpesvirus 8 (HHV-8) is the probable viral etiologic agent for Kaposi's sarcoma. The HHV-8 gene ORF K9 encodes viral Interferon Regulatory Factor (vIRF) which is a protein with low but significant homology to members of the interferon (IFN) regulatory factor (IRF) family. vIRF reduces responses to type I and type II interferons and blocks IRF-1 mediated transcription. In our experimental system the human epithelial cell line, 293 was transfected by a reporter gene plasmid construct in which the human IFN sensitive promoter was followed by a firefly luciferase gene. The RLU values were measured by standard luminometry. Following three independent experiments we concluded that RLU was increased by nearly 5,5 times after IFN-γ treatment for 18 h, while in the presence of v-IRF only an 1,5-fold growth could be detected in the RLU values following the IFN-γ treatment. Based on our results, we could firstly demonstrate the inhibitory effect of vIRF of HHV-8 in a human epithelial cell line following human IFN-γ treatment.

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Hepatitis C and Hepatitis G in Chronic Urticaria. A Case-Controlled Study

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Infection with hepatitis C virus (HCV) is considered to be a possible cause of urticaria. Hepatitis G virus (HGV) is another virus from the same family which is also transmitted by blood transfusions and percutaneous penetration. Its role in pathology is poorly understood. We have performed a case-controlled study to evaluate the frequency of HCV and HGV infection in chronic urticaria. One hundred and ten consecutive patients with typical urticaria lasting for more than 2 mo were included in the Dermatology Department of Strasbourg. None had history of viral hepatitis. During the same period of time, we included 110 age- and sex-matched control patients, none of whom had urticaria, pruritic skin conditions or hepatitis.

The detection of HCV antibodies was made by 3rd generation ELISA. In order to detect early HCV infection, genomic amplification of HCV RNA was carried out, using two methods (a home made RT-PCR and Amplicor HCV*). HGV RNA was also detected by RT-PCR.

HCV antibodies were found in one out of 110 patients with urticaria (0.9%) and in one out of 110 controls (0.9%, p = ns). None of these two HCV+ patients had HCV RNA, and their liver tests were normal. We have not found patients with positive HCV RNA in the absence of HCV antibodies, which could suggest early infection. HGV RNA was detected in two out of 110 patients with urticaria (1.8%) and in two out of 110 controls (1.8%, p = ns). Those four patients were not coinfected with HCV, and all had normal liver tests.

Our study shows that HCV or HGV infection are not significantly more frequent in urticaria than in control patients. Therefore, these virus are not a frequent cause of urticaria. Moreover, the role of HCV infection in our only HCV+ patient is questionable, since HCV RNA could not be detected. There is no argument in favour of the role of HGV in urticaria. The 1.8% rate of HGV positivity found in this study is even lower than the prevalence of this virus in the general French population.

In conclusion, systematic screening for HCV or HGV infection in urticaria is not cost effective.

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Expression of Cellular Prion-Related Proteins (PrPc) by Bovine Epidermal and Mucosal Keratinocytes makes them a Potential Port of Entry for Prion Infection

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Bovine spongiforme encephalopathy is acquired by ingestion of meat and bone-meal derived from scrapie-infected sheep. However, the exact route of transmission of this prion disease remains obscure. To investigate the oral and esophageal mucosa as a possible port of entry for prions we studied expression of cellular prion related protein (PrPc) in skin and mucosa of normal cattle. By immunostaining basal keratinocytes (KC) in mucosa of both locations were positive for this antigen. In addition, also epidermal KC from udder, lip and skin showed anti-PrPc reactivity. In tissue culture KC derived from snout and ear epidermis constitutively expressed PrPc. Deglycosylation with N-Glycosidase F reduced the 25–35 KD protein to a core size of around 25 KD corresponding to the size observed with PrPc from bovine brain tissue under the same conditions. In contrast to what we recently reported for human KC, no distinct regulation of KC-derived bovine PrPc was observed after exposure to TGF alpha or interferon gamma. Our data suggest that squamous epithelial PrPc is easily accessible for prions in cattle and might therefore be the first target in peripheral infection with scrapie contaminated material.

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Epidermodysplasia Verruciformis (EV) Specific Human Papillomavirus in the Processes of Epidermal Repair

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With the use of a very sensitive technique, nested PCR, DNA of various EV HPV types was disclosed in nonmelanoma skin cancers. However, the most oncogenic HPV5 type was found only exceptionally or not at all. In a previous study, unexpectedly, we detected in almost all cases of a large series of psoriasis EV HPV5 DNA and in about one third of cases antibodies against conformational epitope of LI capsid protein of HPV5. Since psoriasis is a benign hyperproliferative disease, we asked a question whether anti-HPV5 antibodies are present also in other conditions associated with epidermal proliferation. We used virus-like particles (VLP) of EV HPV5 and specific ELISA, and for control-ELISA with HPV1 virions. As a model of epidermal repair we chose autoimmune bullous diseases. Of 119 cases studied we found antibodies in 15% of pemphigus vulgaris, 27% of pemphigus foliaceus, 27% of bullous pemphigoid and 20% of dermatitis herpetiformis (DH). To find out what might be the role of autoimmunity in anti-HPV5 antibody formation, we studied autoimmune disorders with cutaneous involvement: 40 patients which systemic lupus erythematosus (SLE) and systemic scleroderma (SSc), *versus* 47 neurological patients without cutaneous lesions (myasthenia gravis, sclerosis multiplex). In SLE and SSc anti-HPV5 antibodies were present in about 17% and 23%, respectively, whereas in neurological disorders they were present only in three of 47 patients. Importantly, only in one of 68 cases of cutaneous neoplasia and warts the results were positive. However, in these patients, as well as in patients with neurological disorders were frequently present antibodies against HPV1. This indicates that in limited cutaneous proliferation (e.g., single carcinomas) or autoimmune diseases without cutaneous involvement there is no generation of specific anti-HPV5 antibodies, whereas production of non EV-related antibodies is preserved. In conclusion, our study showed that epidermal repair, both in bullous diseases and in autoimmune disorders with cutaneous involvement, is associated with anti-HPV5 antibody formation.

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Mannose-Binding Receptors in Killing *Candida albicans* by Human Keratinocytes

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Human keratinocytes (HK) are known to be capable of killing *Candida albicans*. In preliminary studies we proved the involvement of mannose-binding receptors (MBR) in the killing mechanism. Our goal was to give further evidence on the existence of MBRs on HKs and determine their characteristics. Immunohistochemistry with a polyclonal antihuman macrophage mannose receptor antibody exhibited positive staining mainly on the suprabasal HKs. Western-blotting of unstimulated and 365 nm UVA irradiated HKs with the polyclonal antibody revealed the presence of a 75 kDa specific protein. Incubation of cells at 4°C with increasing concentrations of ^{125}I labelled mannosylated bovine serum albumine (^{125}I-Man-BSA) revealed increasing binding to HKs. Unlabelled ligand inhibited the ^{125}I-Man-BSA binding to the cells suggesting, that the binding to HKs was specific. Scatchard analysis of the binding data demonstrated a K_D of 1.4×10^{-8} M and B_{max} of 10.000 receptor/cell. ^{125}I-Man-BSA binding at 37°C showed that the >90% of ligand appeared to be surface-associated. We could demonstrate that HK specifically bound ^{125}I-Man-BSA and this binding could be inhibited by serine-protease trypsin and EGTA. In summary, HKs express MBRs that are different from the macrophage mannose receptor, although they share functional similarities.

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A Comparison of Fusidic Acid Tablets and Flucloxacillin Capsules in Skin and Soft Tissue Infection

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This multicentre, randomised, double blind, parallel group study compared fusidic acid (250 mg, bid) and flucloxacillin (250 mg, qds) was carried out by UK General Practitioners in patients with community acquired skin and soft tissue infections. Treatment was taken for 5 d by all patients and for a further 5 d if the condition remained uncured. In patients cured at the end of treatment, a follow-up assessment was carried out 14 d later.

At the end of treatment the physician rated the condition as "cured/improved" for 182 (76%) of the 240 patients who took fusidic acid and for 189 (80%) of the 233 patients who took flucloxacillin (intention to treat population; $p = 0.16$). Cure was maintained at the follow-up assessment for 94% (138 of 147) and 91% (139 of 153) in the fusidic acid and flucloxacillin groups, respectively. Bacteriological efficacy was rated as a success for 94% (64 of 68) who received fusidic acid and 97% (55 of 57) who took flucloxacillin.

Approximately 30% of patients in each group had adverse events. Diarrhoea was the most common event (fusidic acid 6%, flucloxacillin 5%).

Both treatments were similarly effective and well tolerated. Fusidic acid therefore presents a valuable alternative treatment particularly where penicillin allergy is established or is a possible complication.

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Fusidic Acid and Erythromycin in the Treatment of Skin and Soft Tissue Infection: A Double Blind Study

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This multicentre, randomised, double blind, parallel group study compared fusidic acid (250 mg, bid) and erythromycin (1.0 g, bid) in patients with skin and soft tissue infections. Treatment was taken for 5 d by all patients and for a further 5 d if the condition remained uncured. In patients cured at the end of treatment, a follow-up assessment was carried out 14 d later.

At the end of treatment the physician rated the condition as "cured/improved" for 192 (85%) of the 225 patients who took fusidic acid and for 200 (87%) of the 229 patients who took erythromycin (intention to treat population; $p = 0.52$). Cure was maintained at the follow-up assessment for 95% (151 of 159) and 97% (172 of 177) in the fusidic acid and erythromycin groups, respectively. Bacteriological efficacy was rated as a success for 96% (50 of 52) who received fusidic acid and 97% (56 of 58) who took erythromycin.

The number of patients reporting adverse events was similar for each group (30; 32%). More events were reported for erythromycin (120 v 95). Nausea was the most commonly reported adverse drug reaction (fusidic acid 7%, erythromycin 12%).

Both treatments were similarly effective and well tolerated.

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Neo-Adjuvant Immunotherapy with IL-2 and IFNα in Melanoma Patients Induces Sustained Activation of the Immune System

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Since outcome of patients with metastatic melanoma is generally poor, significant emphasis is put on the development of efficient and well tolerable adjuvant modalities. We have hypothesized that adjuvant treatment strategies might be more successful if initiated before tumor removal that is at a point where tumor antigens are still present for the activation and expansion of tumor specific effector cells. We have conducted a pilot study with 15 patients carrying high risk primary melanomas (pT4) or regional lymph node metastases which received preoperatively as well as postoperatively an immunotherapy consisting of IL-2 and IFNα. The purpose of this study was to examine applicability and toxicity as well as to conduct an immunological monitoring of such a therapy regimen. Patients were treated with nine Mio IFNα IU s.c. on day 1–3 followed by continuous infusion of (A) nine Mio IU IL-2 or (B) 18 Mio IU IL-2 or (C) 12 Mio IU IL-2 sc. on day 4–9. After tumor resection and 1–4 wk of recovery treatment was continued with six Mio IU IFNα 3× per wk s.c. and (A) 4.5 Mio IU IL-2 s.c. 3× per wk or (B) six Mio IL-2 IU s.c. 3× per wk or (C) nine Mio IL-2 IU per d continuously s.c. for 5 d per wk with patients being treated now for up to 8 mo. Side-effects included fever, chills, weakness, edema and skin erythema. Patients in group (C) developed subcutaneous infiltrations at sites of infusion. Overall, toxicity rarely exceeded grade 2 on the WHO scale. After initial decrease due to IFNα, peripheral blood leukocytes as well as eosinophils and CD16+ natural killer cells were significantly increased during the course of the study. While the percentage of CD3+, CD4+ and CD8+ lymphocytes remained unchanged we observed a sustained upregulation of HLA-DR, transferrin-R and IL-2R on lymphocytes. In serum soluble IL-2R, TNFα and neopterin were found to be increased. Immunohistological analyses of the resected tumors showed in most cases the presence of an activated MHC-II+ and TIA1+ T cell infiltrate of both CD8+ and CD4+ cells. A high percentage of tumors were infiltrated by CD1a+ cells and in five cases CD79a+ B cells were found. On melanoma cells MHC-II molecules and the antiapoptotic marker bcl-2 was expressed in 11/15 cases each. Taken together, except in the treatment arm with continuous s.c. IL-2 infusion, this adjuvant therapy has an acceptable toxicity profile and leads to sustained immune activation.

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Valsalva Maneuver is a Useful Method to Detect Skin Autonomous Vascular Regulation

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Local and systemic regulatory mechanisms are responsible for maintaining proper skin blood supply. Failure of the autonomous vascular regulation poses severe problems in diseases like diabetes mellitus, Raynaud's syndrome, etc. We aimed (a) to decide whether baroreflexes (Valsalva maneuver) are appropriate for the initiation of changes in skin blood flow, (b) to determine whether this reflex is mediated by the sympathetic and/or parasympathetic system(s), and (c) to develop a noninvasive method for monitoring autonomous vascular regulation. First the maneuver was performed under physiological conditions to prove its role in modifying skin blood flow. After this we used bretylium locally to neutralize sympathetic activity by presynaptically blocking noradrenalin release. Servo-photoplethysmography was used to monitor haemodynamic changes, while laser Doppler technique was used for measurement of skin blood flow. Without bretylium treatment the cutaneous vascular resistance showed a significant, three-fold increase in the hypotonic phase of the Valsalva maneuver. Bretylium treatment completely inhibited this increase. Our data indicate that baroreflex stimulus results in vasoconstriction in skin arterioles and this vasoconstriction is the result of sympathetic efferent nerve activity. We propose that monitoring of cutaneous vascular resistance over bretylium treated and nontreated skin areas is a convenient and noninvasive way to determine the integrity of cutaneous autonomous regulation of blood supply.

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Ultrasound Induced Skin Damages are not Related to an Increase in Temperature

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We have previously demonstrated that low frequency ultrasound could result in severe burning of exposed hairless rat skin *in vivo*, and resulted in an increase in temperature in the donor compartment. The aim of the present study was to further explain the mechanisms of the skin lesions. We applied 20 kHz ultrasound to hairless rat skin *in vivo* (A group) with two different modes (2.23 W per cm^2 in continuous and 1.78 W per cm^2 in pulsed). We also applied in a second assay (B group) a resistance mimicking thermal elevation in the donor compartment. In both series, temperature was continuously monitored at the surface and within the animal skin. Sonicated area was examined macroscopically, and a biopsy was taken when cutaneous lesions were observed. In each protocols, the temperature at the skin surface was more than 45°C. Within the skin, the temperature increased in few degrees (<4°C) with the resistance and the pulsed mode while with the continuous ultrasound, the increase was higher. In both groups, a slight and transient erythema was observed, presumably due to vasodilatation induced by heating of the skin surface. Two days later, total absence of lesion was observed in the B group, while skin exposed to ultrasound displayed epidermal and dermal necrosis.

Our results show that using two devices resulting in the same increase in temperature at the animal skin surface, lesions were observed only with ultrasound. Thus heating in the donor compartment is not the only explanation of the ultrasound induced lesions. The delayed occurrence of the skin damages must be emphasised. These results might be take into account in understanding the mechanisms and the tolerance of phonophoresis.

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A Unique Topical Vehicle: Increased Bioavailability Imparts Increased Efficacy

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A unique new topical vehicle (a low residue, thermolabile foam) has been developed for the treatment of scalp dermatoses. The objective of the present study was to assess both the comparative bioavailability and therapeutic effectiveness of two corticosteroids [betamethasone valerate (BMV), clobetasol propionate (CP)], in the foam vehicle relative to standard lotion and solution vehicles. Bioavailability was assessed using the *in vitro* human cadaver skin permeation assay. BMV (0.12%) and CP (0.05%) absorption through cadaver skin from the foam vehicle were compared with their absorption from marketed lotion (BMV) and solution (CP) vehicles. Therapeutic efficacy of the same formulations tested in the cadaver skin assay were also evaluated in two separate studies of patients with scalp psoriasis. Treatment was twice daily for either 2 (CP) or 4 (BMV) wk.

At the end of treatment, patients rated as completely clear or almost clear of disease were: (1) 72% on BMV foam *versus* 47% on BMV lotion, and (2) 74% on CP foam *versus* 63% on CP solution. Cadaver skin permeation data demonstrated a two-fold (CP) and six-fold (BMV) increase in the rate of absorption from the foam *versus* the liquid vehicles.

The bioavailability and efficacy of corticosteroids such as BMV and CP are greatly enhanced when incorporated in the foam vehicle.

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13-*cis* Retinoic Acid Inhibits Sebocyte Proliferation Through Intracellular Isomerization into All-*trans* Retinoic Acid and Binding to Retinoic Acid Receptor

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Despite its biological activity on human sebocytes *in vivo* and *in vitro*, 13-*cis* retinoic acid does not bind cellular retinoic acid-binding proteins and has a low affinity for nuclear retinoid receptors. It has been therefore postulated that 13-*cis* retinoic acid is a pro-drug and exhibits its activity through isomerization to all-*trans* retinoic acid. In this study, intracellular conversion of 13-*cis* retinoic acid to 2- to 15-fold higher levels of all-*trans* retinoic acid at 12–72 h was assessed by high performance liquid chromatography. In contrast, intracellular all-*trans* retinoic acid was converted to barely detectable 13-*cis* retinoic acid amounts. Co-incubation of 13-*cis* retinoic acid and AGN193109, a potent antagonist of the retinoic acid receptor, resulted in a slight delay of 13-*cis* retinoic acid uptake and in a late intracellular increase of retinoic acid concentration compared to incubation with 13-*cis* retinoic acid alone. In parallel experiments, 13-*cis* retinoic acid significantly reduced sebocyte proliferation at 10^{-7} M with approx. 30–40% inhibition after 6 d and with 50% after 9 d. AGN 193109 antagonized the antiproliferative activity of 13-*cis* retinoic acid in a dose-dependent manner; at 10^{-8} M, it completely abolished the 13-*cis* retinoic acid effect. Similarly, AGN 193109 neutralized the antiproliferative effect of all-*trans* retinoic acid at a 10-fold higher concentration. In contrast, CD3159 (LG100754) and CD3700, retinoid X receptor antagonists, did not affect the 13-*cis* retinoic acid-induced inhibition of sebocyte proliferation. In conclusion, the inhibitory effect of 13-*cis* retinoic acid on sebocyte proliferation obviously results from its intracellular conversion to all-*trans* retinoic acid and subsequent binding of the latter to the nuclear retinoic acid receptor.

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Photodynamic Therapy on Pig Skin with Intracutaneously Injected Delta-Aminolevulinic Acid as a Photosensitizer

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Photodynamic therapy (PDT) with topically applied delta-aminolevulinic acid (d-ALA) in cream as a precursor-photosensitizer can be used successfully for treatment of superficial growing cutaneous (pre)malignancies. In nodular growing tumors d-ALA will penetrate insufficiently. We developed a solution containing d-ALA for intracutaneous injection with the intention to enhance the effectivity of PDT for nodular growing skin tumors.

We performed animal studies to investigate toxicity and effectivity of d-ALA solution. D-ALA in different concentrations/volumes was injected intracutaneously into normal skin of six Dutch pigs to find out the doses that can be used safely without leading to necrosis. At fixed intervals after injection the level of fluorescence of the photosensitizer was measured for each dose and compared to d-ALA in cream. These levels may indicate the effectivity of the therapy. We looked for the time-interval at which the fluorescence was highest for each dose. Three hours after injection each testarea was irradiated with 100 mW per cm^2–60 J per cm^2. Because oxygen is a rate-limiting factor in PDT, we also evaluated the effect of two fractionated light doses to create the facility for new oxygen supply to the irradiated tissue. Skin biopsies were taken to examine the histological changes after PDT.

The dissociation of d-ALA in water seems to depend on the pH of the solution, but not on the con-centration of d-ALA. For intracutaneous injection a pH of 5.0 was chosen. D-ALA concentrations up to 5% can be used safely for injection. Because an injection of 0.5 ml with a d-ALA concentration of 1% shows higher levels of fluorescence compared to d-ALA cream, this concentration should be sufficient to be effective. After injection of concentrations of 1% or more the effective level of fluorescence is reached within 2 h (4 h for d-ALA in cream), so the uptake of d-ALA is the rate-limiting factor and not the conversion of d-ALA into the active photosensitizer. The effect of the fractionated irradiation so far was equal to the single illumination. According to the results of the animal study we expect that PDT with intracutaneously injected d-ALA can be an effective treatment for nodular growing skin-tumors, with deeper effects and shorter injection-illumination interval compared to d-ALA in cream.

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The Guinea Pig (*Cavia Porcellus*) as a Model of the Itch Sensation

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Itch is a distressing symptom and at present investigations into its basis are hampered by the lack of generally accepted animal model.

Two methods were employed in the present study to induce scratching in the guinea pig, histamine iontophoresis and dinitrochlorobenzene (DNCB) contact sensitivity. Solutions were applied and the animals were videoed for up to 33 h. 30 minute periods at 3 h intervals were analysed to give an overview of scratch behaviour then analysis was restricted to the period of greatest scratching activity. The number of scratch bouts, number of scratch movements, activity and other grooming behaviours were recorded.

The overview of scratching behaviour for DNCB contact sensitivity showed controls had a higher number of scratch bouts compared to experimental animals, the last significant difference being 15 h after solution application ($p < 0.01$, $n = 7$ whereas for histamine the greatest difference was found to be in the first 30 min period. There was clear evidence of a dose response between dose of DNCB and scratch bout numbers ($p < 0.01$, $n = 28$). By contrast it was the first 20 min after iontophoresis that experimental animals had a higher number of scratch bouts than controls, though this was not significant ($p = 0.117$, $n = 7$). Neither histamine iontophoresis or DNCB contact sensitivity appeared to induce differences in other behaviours measured.

This shows that it is possible to induce chronic and acute scratching behaviour in Guinea pigs with the behavioural response being similar in both cases.

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Actigraphs as an Assay for Itch and Scratch Behaviour: A Pilot Study

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This study investigates the possible use of limb meters (Actigraphs) to measure scratching, the behavioural response to itch, in patients in their own home.

The limb meters used in this study contain a piezoelectric beam that produces a voltage when moved in any plane. The voltage is recorded at a sampling rate of 10 Hz and stored in 2 s epochs. Preliminary investigation using simulated scratch movements showed that when a setting of low amplification, high sensitivity, recording in the range 2.0–9.0 Hz was used movements could be isolated and traced to particular limbs. Patients with itchy dermatoses were monitored overnight and compared to controls.

There was a significant difference between wrist and ankle mean movement scores in patients with itch ($p < 0.05$, $n = 16$) and this difference was not observed in control subjects. No significant difference between mean movements of right and left hands in either controls or patients was found. There was however, a significant difference between the mean hand movements of itchy patients and the mean hand movements of the control subjects ($p < 0.05$, $n = 20$) but there was no difference between mean leg movements of itchy patients and mean leg movements of controls.

These results suggest that it is possible to determine differences in the night-time movement patterns of patients suffering pruritus and control subjects with the use of actigraphs with the ratio of wrist to ankle movement being a useful guide to severity of scratch.

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Hypothalamus-Pituitary-Adrenal Axis Suppression by Clobetasol Propionate 0.05% Ointment used in Bullous Pemphigoid

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Some studies have demonstrated the potential for Hypothalamus-pituitary-adrenal (HPA) axis suppression from high-potency corticosteroids topical agents. We report a prospective study evaluating the HPA axis in Bullous Pemphigoid (BP) patients treated by clobetasol propionate ointment

Methods: Inclusion criteria were (1) clinical and histological examination compatible with BP. (2) Linear deposits of IgG and/or C3 in the Basement Membrane Zone by DIF. (3) treatment by clobetasol propionate 0.05% ointment 40 gm per d. Doses were decreased 14 d after epithelialisation. (4) Serum cortisol level and ACTH test were performed on days 0, 8 and 30 of treatment.

Results: Twenty-two patients (15 women, seven men), mean: 81.7 y old were included (Jan. 1996 to Feb. 1999). The mean daily number of new bullae was 33 (1–190).

Day 0: serum cortisol levels were all in the normal range. ACTH-test was always positive (14 patients)

Day 8: serum cortisol levels dramatically decreased in 17/18 patients (mean 52 nmol per liter (25–281)) ACTH-test was negative in 12 of 15 patients.

Day 30: serum cortisol levels dramatically decreased in 10 of 13 patients (mean 74 nmol per liter (15–179)) ACTH-test was negative in eight of 13 patients.

Comments: This study demonstrates a suppressed HPA axis by a daily application of 40 gm of clobetasol propionate 0.05% ointment in BP patients. HPA axis suppression was powerful and early.