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ABSTRACTS

32nd Annual European Society for Dermatological Research (ESDR) Meeting 2002

September 19-21, 2002, Geneva, Switzerland

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001 [Oral 022]

Anti-Idiotype Vaccination Induces T Cell Responses Against Conserved Framework Sequences Rather than the Hypervariable CDR3 Region

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The idiotype region of the T cell receptor (TCR), because of its restriction to the malignant T cell clone, is generally seen as an ideal tumour antigen for vaccination therapy of T cell lymphomas. Similarly, the variable regions of immunoglobulins are preferred targets for antitumour vaccinations in B cell lymphomas. Consequently, anti-idiotype vaccination is the major strategy for therapeutic vaccination in cases of cutaneous and systemic lymphomas. To identify likely targets of these strategies, we have subjected all 74 completely sequenced TCRVb to computational analyses for T cell epitopes taking into consideration the protein chemical constitution of these molecules, and algorithms for the prediction of proteasome cleavage (Holzhütter, 1999) and for MHC binding (SYFPEITHI). To include epitopes that are generated from proteasome products by subsequent trimming, octa to deca peptides were predicted from the protein sequences and analysed further for peptides that conform to the MHC allele-specific anchor motifs of HLA-A1, -A2.1, -A3, -B7, -B8 as the most frequent HLA alleles in the middle European populations. Overall, these analyses yielded a predictably large number of potential epitopes. However, only a relatively small number of these epitopes scored highly for MHC binding thus indicating that the variable domain of T cell receptors is not an efficient T cell antigen. Most strikingly, the hypervariable idiotype regions are almost completely devoid of potential T cell epitopes which almost all reside in conserved framework sequences. This out-come is in good agreement with the protein chemistry of the proteins and of MHC-binding peptides. Hydrophobic amino acids are preferred in MHC-binding peptides. Such amino acids are abandon in the framework regions that constitute protein cores but are largely lacking in the exposed hypervariable loops, in particular in the CDR3 sequences. These findings might explain why almost all verified TCR T cell epitopes are framework sequences. In conclusion, vaccinations with TCR sequences will preferentially induce TCR family specific and not tumour-specific immune responses.

002 [Oral 025]

Induction of a Caffeine-Sensitive S-Phase Cell Cycle Checkpoint by Psoralen Plus Ultraviolet A Radiation

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Induction of interstrand crosslinks (ICLs) in chromosomal DNA is considered a major reason for the antiproliferative effect of psoralen plus ultraviolet A (PUVA). It is unclear as to whether PUVA induced cell cycle arrest is caused by ICLs mechanically stalling replication forks or by triggering cell cycle checkpoints. Cell cycle checkpoints serve to maintain genomic stability by halting cell cycle progression to prevent replication of damaged DNA templates or segregation of broken chromosomes. In response to DNA damage, the caffeine-sensitive kinases ATM or ATR phosphorylate and thereby activate the checkpoint kinase Chk1. Activated Chk1 phosphorylates Cdc25 dual-specificity protein phosphatases which promote cell cycle transitions by dephosphorylating cyclin-dependent kinases. Phosphorylation of Cdc25 phosphatases inhibits their function or targets them for degradation. Here we show that HaCaT keratinocytes treated with PUVA arrest with S-phase DNA content. Cells that had completed DNA replication were not perturbed by PUVA and passed through mitosis. PUVA induced rapid phosphorylation of the Chk1 checkpoint kinase at Ser345 and a concomitant decrease in Cdc25A levels. The decrease of Cdc25A levels and the S-phase arrest were both abolished by caffeine, suggesting that active checkpoint signaling rather than mechanical blockage by ICLs causes the PUVA induced replication arrest.

003 [Oral 026]

Drebrin – an Actin-Binding Protein Involved in Regulations of Cellular Protrusions and Cell Motility

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Drebrins are actin-binding proteins initially identified in – and thought to be specific for – neuronal cells. Recently, we detected the drebrin isoform E2 as a widespread, although not ubiquitous, component of the actin cytoskeletal system of various cell types. In cells forming adhering junctions drebrin is associated with the submembranous actin microfilament system whereas in motile cells it is accumulated in lamellipodia, filopodia and other cellular protrusions. Here we present results indicating that drebrin accumulation at adhering junctions and in cellular processes plays a role in the physiology and pathophysiology of human skin. Using immunocytochemistry and immunoblotting, we have identified drebrin in a number of skin-specific cell types, including keratinocytes, melanocytes and Merkel cells. Certain skin tumors, notably basal cell carcinomas and melanoma metastases, seem to contain more drebrin. *In vitro* wound healing models show enhanced drebrin immunostaining in keratinocytes migrating into the wound. Analysis of the physical state of drebrin by sucrose gradient fractionation and immunoprecipitation has revealed drebrin-containing particles ("drebrosomes") including some in which drebrin is associated with actin and partly also with certain membrane-associated actin binding proteins. We conclude that drebrin is involved in the regulation of the actin cytoskeleton in cellular processes and at membranes, possibly serving as a membrane-cytoskeleton linker. Drebrin is recruited to sites of protrusions in situations of increased cellular motility, suggesting a role in the regulation of actin during cell migration.

004 [Oral 027]

Cx26 Null Mutation Affects the Development of Human Epidermis *In Vitro*

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Recently, connexin (Cx) mutations of Cx26 have been identified in hereditary deafness associated or not to abnormal epidermal differentiation. Among this mutation, the most frequent, is the Cx26 null mutation (35delG homozygous). The Cx26 is highly expressed in epidermal hyperproliferative state (psoriasis, reepithelialisation), in hair follicles or in mucosa. Surprisingly, the patients bearing the Cx26 null mutation did not have an abnormal skin phenotype. To study this paradox, we have analysed the proliferation and migration of keratinocytes from 5 patients bearing a Cx26 null mutation (35delG homozygous) and studied their ability to form *In vitro* a so called epidermal equivalents. The absence of Cx26 resulted in a reduced thickness of epidermal equivalents ($n = 60$, $p < 0{,}0001$), an alteration, which was not observed in 5 healthy volunteers ($n = 75$). The colony forming efficiency of keratinocytes from homozygous 35delG ($n = 40$) is reduced ($p < 0{,}0001$) as compared to 4 heterozygous ($n = 50$) and control patients ($n = 50$). By time lapse videomicroscopy, we demonstrated that the migration of the mutated keratinocytes was dramatically reduced as compared to normal keratinocytes. By fluorescent dye microinjection and scrape loading, we were unable to demonstrate a difference in gap junction intercellular communication. The mRNA coding for Cx30, 30.3, 31, 31.1, 43 was detected by RTPCR. *In vivo*, the surprising aspect of the 35delG mutation is that apart a neurological deafness, the absence of Cx26 seems not to cause gross functional abnormalities in others organs, where this connexin is normally expressed. In contrast, we demonstrated that, in an *In vitro* controlled environment, the absence of Cx26 reduced the colony forming efficiency and migration of keratinocytes, which could be involved in the impaired stratification of the epidermal equivalents. We can speculate that, even if the repithelialisation is perturbed *In vivo*, these alterations revealed *In vitro* are not measurable by clinical observations.

005 [Oral 032]

Keratin5-Cre/lox-P Mediated Deletion of Vascular Endothelial Cell Growth Factor Severely Compromises Mammary Gland Development and Function

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VEGF is essential for embryonic blood vessel development, and in the adult remains the major regulator of angiogenesis not only during patho-physiological processes, but also during the female reproductive cycle. We have previously shown that keratin5 driven, Cre/lox-P mediated deletion of VEGF in epidermal keratinocytes delays wound healing and prevents tumor growth. As an appendage of the skin, mammary gland epithelium also expresses keratin 5, and VEGF is also deleted in this tissue in K5-Cre VEGF flox/flox mice. Such females bear few live young, probably due to genomic deletion of the VEGF gene caused by Cre activation during fertilization, confirming the necessity for VEGF during embryonic development. Mammary gland ducts of pubescent virgin K5-Cre VEGF flox/flox mice are shorter, have fewer branches and a smaller diameter compared to controls, and pups which are reared by mutant adult females are growth retarded, and display reduced amounts of milk in their stomachs. Immunohistochemistry of mammary glands from mutant lactating females reveals a reduced number of mammary alveoli, lined by droplet-containing cells that are not present in control mice. We conclude that lack of VEGF production in mammary epithelium results in severely compromised development and function.

006

Seborrheic Keratoses Contain Higher Levels of Lipid Peroxides and Less α-Tocopherol than Normal Epidermis

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Background: Seborrheic keratosis is a common and benign lesion of the epidermis; its aetiology is still unknown. The skin is constantly exposed to various environmental factors (UV, oxidants) and is therefore susceptible to suffer from oxidative stress. α-tocopherol (vitamin E) plays an important role in the antioxidant defence system of the epidermis and functions *In vivo* as a protector against lipid peroxidation. Objectives: We assessed the oxidative stress by comparing α-tocopherol and lipid peroxide concentrations of seborrheic keratosis with those of the epidermis of healthy volunteers. Methods: 32 Seborrheic keratoses were collected by vaporisation with liquid nitrogen followed by shaving, and 17 epidermal samples were collected from plastic surgery for controls. α-tocopherol was analysed by high-pressure liquid chromatography, and lipid peroxides were assayed by ferrous oxidation followed by xylenol orange complexometry. Results: Seborrheic keratoses showed a lower concentration of α-tocopherol (13.2 ± 2.4 nmol per g) as compared to healthy control epidermis (38.3 ± 5.9 nmol per g) ($p<0.001$), as well as a higher concentration of lipid peroxides (504.1 ± 73.4 and 212.0 ± 27.0 nmol per g for seborrheic keratosis and healthy control epidermis, respectively) ($p<0.001$). The mean DNA content was comparable between seborrheic keratosis and the epidermis (4.05 ± 0.46 mg per g and 4.17 ± 0.35 mg per g respectively). Conclusion: Seborrheic keratoses were shown to exhibit a higher oxidative state than the epidermis of healthy volunteers. We don't know whether this is a cause or a consequence of the lesions, but this can be added to the growing list of the pathologies associated with an oxidative stress.

007

Macrophage Metalloelastse (MMP-12) and 92-kDa Gelatinase are Overexpressed in Nodular Kaposi's Sarcoma

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Kaposi's sarcoma (KS) is an angioproliferative tumor that exists in several clinical and epidemiological forms. They all share same histological features and are associated with infection by human herpesvirus 8 (HHV8). Histologically KS is characterized by growth of spindle cells and a lymphomononuclear infiltration. The classic KS shows tendency to skin lesions while AIDS – associated KS can widely disseminate to other organs. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes, most of which have been implicated in cancer growth and angiogenesis. Their role in KS is not well studied except that MMP-2 and MMP-9 have been reported in KS cell cultures by Northern and Western analysis. In fact synthetic MMP inhibitors are currently in clinical trials as a therapy for KS. Our initial aim was to study putative expression of macrophage metalloelastase (MMP-12) in KS. We have previously shown that in skin cancer the amount of MMP-12 positive macrophages correlates with good prognosis while that produced by epithelial cancer cells implicates aggressive phenotype. Expression of other MMPs was also studied by immunohistochemistry or *in situ* hybridization in 28 classical and 4 HIV-associated Kaposi's sarcoma. 11 specimens represented patch stage, 6 plaque lesions and 15 nodular tumor stage. MMP-12 was expressed in 12/32 specimens by CD-68 positive correlating with nodular stage. MMP-2 was found in fibroblasts surrounding the tumor nodules but not in spindle cells. MMP-9 was expressed by neutrophils infiltrating into tumor and by macrophages and endothelial cells inside the tumor nodule. MMP-19 was detected in endothelial cells of the tumor nodule and occasionally in fibroblasts and macrophage-like cells. Our results suggest that selective inhibitors of MMP-12 and MMP-9 could be beneficial for treating Kaposi's sarcoma

008

Expression of 4a Carbinolamine Dehydratase and Hepatocyte Nuclear Factor 1α and Transcription of Albumin in the Human Epidermis

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Recently protein expression as well as enzymatic function of 4a carbinolamine dehydratase (PCD) has been demonstrated in the human epidermis *in situ* and in cell extracts from suction blister roofs. Since this enzyme also acts as a dimersation cofactor of hepatocyte nuclear factor 1α (DCoH), it was tempting to look for this function of PCD/DCoH. DCoH and hepatocyte nuclear factors 1α (HNF-1α) transcribe a number of genes, including albumin. Using immunohistochemistry with antibodies directed against human PCD/DCoH, HNF-1α and human albumin, we were able to demonstrate the presence of PCD/DCoH, HNF-1α and albumin in human melanocytes and keratinocytes *In vitro* and *in situ*. Applying the double immunofluorescence technique, we demonstrated colocalisation of PCD/DCoH and HNF-1α in the nucleus and in the cytosol of melanocytes and keratinocytes. Furthermore, we demonstrated also the colocalisation of HNF-1α and albumin in both cell types and *in situ*. The presence of albumin was confirmed by electrophoresis and Western Blots. The results of this study show for the first time the expression of HNF-1α in the human epidermis together with DCoH and albumin, supporting transcription of the albumin gene in melanocytes and keratinocytes.

009

Regulation of the Lipoprotein Lipase Activity in 3T3-L1 Adipocytes Using an Oxazolidinone Derivative

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The Lipoprotein Lipase (LPL) which is present in the adipose tissue is an enzyme which plays a main role in the the adipocyte's lipid storage. The LPL is synthesized by the adipocyte, secreted in the interstitial space and carried to the capillary endothelial cells where it binds with glycoaminoglycans. It thus becomes responsible for the hydrolysis of endogeneous triglycerides (VLDL) and exogeneous triglycerides (chylomicrons) and generates free fatty acids and monoacylglycerol during circulation. As soon as they are released, the fatty acids can be caught by the adipoctye, re-esterfied and stored in triglyceride form. As the series phenomena suggests that the LPL plays a role in the development of the adipose tissue, we thought it would be interesting to identify the potential regulators of this enzyme. Amongst the components tested, one molecule (sphingosine biomimetics: oxazolidinone) was selected for its ability to modulate the activity of the LPL secreted by the 3T3-L1 adipocytes in culture. The secretion level of the LPL was measured by radioisotopic dosage of the enzymatic activity. It clearly showed that the molecule has an obvious dose-dependant inhibiting effect on the LPL activity. This effect reaches its height after 48 h of treatment by the molecule. Furthermore, this molecule is a moderate inhibitor of the adipocyte differentiation process (dosage of the specific G_3PDH activity) and slightly reduces the cellular triglyceride content. The molecule thus appears to be of potential interest in modulating the importance of subcutaneous adipose deposits.

010

Tight Junctions and New Compositionally Related Structures in Epidermis and Other Stratified Epithelia

L. Langbein, C. Grund, C. Kuhn, S. Praetzel, J. Kartenbeck, J. M. Brandner, I. Moll, and W. W. Franke
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The occurrence of tight junction (TJ) structures and the spatial arrangement of TJ proteins in stratified epithelia have long been controversial. Therefore, we have systematically examined the localization of TJ proteins (occludin, claudins 1 and 4, ZO-1, cingulin and symplekin) in diverse stratified epithelial tissues, including skin and cell cultures by electron microscopy and immunocytochemistry at both the light- and the electron microscopic level. We have found an unexpected diversity of TJ-related structures of which only those showing colocalization with occludin, the most restricted transmembrane TJ-protein, are presented here. While in the epidermis as in all the other epithelia a true, probably continuous occluding TJ zone is restricted to the uppermost living cell layer(s), TJ-related junctions are abundant in several or even the majority of the suprabasal cells. Interfollicular epidermis contains in the *str. granulosum* extended TJ structures, which can also be traced, at least, through the Henle cell layer of hair follicles. Besides "classical" TJs, however, we have noticed TJ protein-positive junctional structures, represented mostly by broad, ribbon-like membrane contacts that often appear pentalaminar, with an electron-dense middle lamella ("lamellated TJs", *coniunctiones laminosae*) and numerous junctions with a 10–30 nm dense intermembraneous lamina ("sandwich junctions"; *iuncturae structae*). Corresponding TJs and compositionally related junctions are also formed in cell cultures during stratification. These junctions are mostly in close vicinity to and in their amount next to desmosomes and obviously part of the epithelial barrier.

011

Diversity of Desmoplakin Expression in Basal Palm Keratinocytes: A Possible Clue to the Identification of Epidermal Stem Cells

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Desmosomes are the most abundant intercellular junctions in skin and play a pivotal role in epithelial sheet formation and maintenance of tissue integrity and function. The expression of desmosomal proteins in skin varies according to the body site and differentiation status of the epidermis. Using immunofluorescence and *in situ* hybridisation, we found that desmoplakin expression varied in the basal layer of normal palm skin *In vivo* as well as in the cultured keratinocytes of the same phenotype. Cells located at the bottom of deep rete ridges, the region of putative stem cells, showed a low level of desmoplakin expression, while those at the side of rete ridges or above the dermal papillae, expressed abundant desmoplakin. Small keratinocytes also expressed less desmoplakin *In vitro*, compared with larger cells. We hypothesized that putative epidermal stem/progenitor cells express fewer desmosomes. To test this, we separated trypsin-dissociated basal palm keratinocytes into two populations according to their rapid (20 min) or slow (20 min+) ability to adhere to type IV collagen, a selection procedure corresponding to the cell's β1 integrin level. We used unseparated cells as the control. We further examined desmoplakin expression between these two populations by Western blotting and immunofluorescence and found that cells with a high β1 expression appeared to have a low desmoplakin expression, and vice versa. Similar expression profiles between the two cell populations were also observed for other major desmosome proteins including desmoglein, desmocollin, plakoglobin and plakophilin1. In conclusion, keratinocytes with a high β1 integrin expression, the putative stem cell enriched population, express a low level of desmosomal proteins, and, by inference, are "desmosome sparse". This finding may provide a novel marker for the purification of putative epidermal stem cells.

012

Dynamic Characterisation of the Molecular Events Controlling Dermal Fibroblasts Proliferation During Wound Healing

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Skin wound healing is a complex process allowing the reconstruction of epidermal and dermal lesions caused by mechanical, chemical or thermal agents. Many genetic or acquired defects can perturb the normal wound healing process, leading to skin pathologies like hypertrophic scars or keloïds, in which fibroblasts are characterised by an important abnormal proliferation. However, the molecular abnormalities at the origin of these pathologies are still unknown notably because the experimental systems used to study wound healing do not allow a molecular approach of the process. To circumvent this hurdle, we have developed an original device that performs calibrated injuries of great length within confluent cell cultures or reconstructed dermis and skins and thus enables the detection of a wide range of molecular events activated during *In vitro* wound healing. Using this system we demonstrate that mechanical lesions performed within dermal fibroblast cultures lead to the phosphorylation of pRb and p107, the stimulation of CDK4 and CDK2 kinase activity and the cells commitment into S phase. Two major events play a key role in these different processes: (1) the stimulation of cyclin D1 expression, and (2) the decrease in the expression of the CDKs inhibitor $p27^{KIP1}$. First, we propose that the stimulation of cyclin D1 expression could trigger $p27^{KIP1}$ relocalisation from cyclin E/CDK2 to cyclin D1/CDK4 complexes, leading to cyclin E/CDK2 complexes activation and induction of genes necessary to the cell commitment in S phase. On the other hand, we bring evidences indicating that $p27^{KIP1}$ is phosphorylated by cyclin A/CDK2 complexes and degraded *via* a proteasome-dependent pathway. In conclusion, we propose here a coherent scheme describing, for the first time, the cascade of events that initiate and regulate the fibroblasts proliferation during the dermal repair *In vitro*.

013

Induction of Cytochrome P450 Enzyme Activity by UVB and Xenobiotics in Normal Human Keratinocytes and Melanocytes

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Cytochrome P450 (CYP450) plays a major role in the bioactivation of procarcenogenesis in target tissues and the expression of this enzyme is an important determinant of human susceptibility to cancer. Relatively little is known about the overall role of CYP450 in the metabolism of xenobiotics or endogenous cellular compounds in the skin. The aim of this study was to analyse the expression of cytochrome enzymes in proliferating human keratinocytes and melanocytes after exposure to UVB radiation and to three classical cytochrome inducers such as: β-naphthoflavone (BNF), 3-methylcholanthrene (MC), phenobarbital (PB). We investigated 7-ethoxyresorufin O-deethylase (EROD) (which is CYPP450 1A1 dependent) and 7-pentoxyresorufin O-depenthylase (PROD: CYP450 2B1 dependent) activities. Normal human keratinocytes were cultivated in Dulbecco's modified Eagle's medium/Ham's F12 or with KGM serum-free medium. Melanocytes were grown in Medium 154. At confluency cells were incubated with inducers or irradiated with different doses UVB. At different times after treatments, cells were harvested for *In vitro* measurement of CYP450 induction. The microsomal fraction was studied by Western blot analysis. Low, but measurable levels of CYP activity were detected in both basal and differentiating keratinocytes. The MC- induced EROD activity was up to 4 fold higher when compared with BNF induced activity. UVB exposure resulted in a dose-dependent (10–75 mJ) and time dependent (4–24 h) induction of CYP450 1A1 for keratinocytes and CYP450 2B1 for melanocytes. Immunoblotting assay showed expression for CYP450 1B1 for both keratinocytes and melanocytes. Proadifen, an inhibitor of CYP450-monooxygenase, led to a significant decrease in EROD activity. The results of the present study clearly show that irradiation with UVB is capable of modifying the activity of CYP450 isoenzymes not only in keratinocytes but also in melanocytes. These experimental findings stress the value of epidermal cell culture for parmacotoxicological studies of topical agents used in dermatology.

014

Gap Junctions and Wound Healing

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Gap-junctions (GJ) are cell-cell junctions allowing the communication and coordination of adjacent cells by the exchange of small molecules, e.g. second messengers. They play important roles in proliferation, migration and differentiation of many cells, processes which are all included in wound healing. We have examined the fate of various gap junction-proteins, i.e. connexins 26, 30 and 43, during spontaneous wound healing and after the transplantation of keratinocytes by using a wound healing model. Moreover we investigated the influence of gap junction-inhibitors on wound healing. We show the lack of all three GJ proteins at the wound edges at the beginning of wound healing and an up-regulation of Cx 26 and 30 in the course of reepithelialisation. Transplanted keratinocytes are positive for Cxs already at very early time points after transplantation. The inhibition of GJ communication by inhibitors resulted in a delay in wound healing. These results argue for an important role of the coordinated synthesis and function of GJ proteins and channels during wound healing. The putative implication on nonhealing wounds is discussed.

015

Biological Effects of Hormonal Aging on Human Sebocytes *In Vitro*

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Hormones have been suggested to exhibit a profound influence on sebaceous gland aging. In order to elucidate this hypothesis, we investigated the biological activity of hormones at levels representative for different age groups in females on SZ95 sebocytes *In vitro*. First, growth hormone receptor (GHR), insulin receptor (IR), insulin-like growth factor I receptor (IGF-IR), androgen receptor (AR), estrogen receptor–α and -β (ERα and ERβ) were examined by highly sensitive RT-PCR and Western blotting in SZ95 sebocytes and were found to be expressed. SZ95 sebocytes incubated with several hormones at levels similar to those found in postmenopausal women showed significantly lower content of neutral lipids (–10%), as detected with the nile red fluorescent lipid microassay, compared to the lipids detected in sebocytes incubated with hormones at levels representing 20-year-old-women after a 2-d treatment. Polar lipids, cell proliferation estimated with the MUH fluorescent assay, and toxicity measured by the lactate dehydrogenase colorimetric assay remained unchanged. SZ95 sebocytes also responded to IGF-I, as a single agent, at the lower concentrations of postmenopausal vs. young women with a marked reduction of neutral (–20%) and polar (–8%) lipids. Cell proliferation rates were similar after 4 d of treatment. Postmenopausal levels of testosterone also reduced neutral lipids compared to the levels of young women (–12%). In contrast, GH, 17β-estradiol, progesterone and DHEA, as single agents, in concentrations of postmenopausal women did not change lipid synthesis or proliferation of SZ95 sebocytes under hormone concentrations of young women. These results suggest that human sebocytes express several hormone receptors, especially GHR, IR, IGF-IR, AR, ERα and ERβ and are therefore susceptible to multiple hormonal effects. Among these hormones, IGF-I, and at a lower magnitude testosterone, maintained as single agents the effects of age-relevant hormone mixtures on sebaceous lipids.

016

The Role of Postmitotic Differentiated Fibroblasts in Retinoic Acid Induced Synthesis of Procollagen I in Photodamaged Skin

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This study investigated the role of differentiated fibroblasts in the production of newly synthesised collagen during the repair of photodamaged skin by retinoic acid. A clinical study showed that daily application of 0.05% retinoic acid, for 14 or 28 weeks, to moderately photodamaged forearms of healthy volunteers increased the dermal levels of procollagen I (PCI). A heterogeneous pattern of expression for PCI was observed surrounding papillary dermal fibroblasts. It has also been reported that a heterogeneous population of mitotic (defined as type 1) and postmitotic (defined as types 2 & 3) differentiated cells exists in fibroblast cultures (Alaluf *et al* Differentiation 2000, 66:147) and in human dermal fibroblasts derived from photodamaged skin. Using a dot blot protein assay to measure PCI secretion, we found that cultures with elevated proportions of type 3 fibroblasts closely associated with increased levels of PCI production ($r^2 = 0.73$, $p < 0.002$). Immunofluorescence and Western blot analysis showed that type 3 fibroblasts express approximately 3 fold higher levels of PCI and higher levels of the TGFb receptor type II compared to type 1 fibroblasts. Treatment of cultured dermal fibroblasts with retinoic acid increased the proportion of type 3 fibroblasts. Treatment of photodamaged skin with retinoic acid also led to increased TGFβ1 protein expression in the epidermis and dermis compared to placebo as measured using immunohistochemistry. Taken together these results suggest that retinoic acid may stimulate the repair of photodamaged skin by promoting fibroblast differentiation to post mitotic collagen producing (type 3) cells and by stimulating PCI synthesis in these fibroblasts through a TGFb1 pathway.

017

Semiquantitative Detection of Lipids by a Quick Fluorescence Assay and Their Regulation Through Androgens, the PPAR-Ligand Linoleic Acid and Hydrocortisone in Human Sebocytes

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A major characteristic of sebaceous gland cell (sebocyte) differentiation is the intracellular accumulation of neutral lipids. To detect these lipids, several techniques are available which are either time consuming or expensive. A second important matter is the distinction between neutral and polar lipids. While the fluorescent dye nile red is capable to detect both lipid classes, detection of lipids by flow cytometry using nile red staining is limited by the fact that adherent growing mature sebocytes are destroyed after cell dissociation and proliferating, undifferentiated and early differentiated sebocytes can only be evaluated. Labelling the cells on chamber slides eliminates this technical disadvantage but does not allow quantification of lipids. To resolve this problem, SZ95 sebocytes were directly labelled with nile red (1 μg per ml) in 96-well plates after 24 and 48 h of treatment and the resulting fluorescence was detected by a spectrofluorometer at different wavelengths for neutral (excitation 485 nm, emission 565 nm) and polar lipids (excitation 540 nm, emission 620 nm). SZ95 sebocytes were treated 2 d after seeding with testosterone (10^{-5}–10^{-4} M), dihydrotestosterone (DHT, 10^{-6}–10^{-4} M), the PPAR-ligand linoleic acid (LA, 5×10^{-5}–10^{-4} M) and hydrocortisone (HC, 10^{-9}–10^{-6} M) under serum-containing conditions. Fluorescence values were referred to cell numbers per well measured by the MUH-assay; cytotoxicity was evaluated by the lactate dehydrogenase assay. Similar cell numbers and no significant cytotoxicity were detected after 24 and 48 h of treatment with all compounds. In contrast, a dose-dependent increase of neutral lipids was found after cell incubation with testosterone and DHT (~ 10–50%) and a dramatical increase with LA (~ 50–600%). A decrease of neutral lipids occurred after incubation with HC (~ –15% at 10^{-7} M) for 48 h. LA which was the strongest enhancer of neutral lipids, also increased slightly the polar ones (~ 10–60%). In conclusion, we developed a simple and fast method to screen different drugs for their capacity to regulate synthesis of total, neutral and polar lipids in adherent cell cultures.

018

Regulation of Apoptosis of Human Mast Cells by Anticancer Drugs

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Agressive mastocytosis and mast cell leukemia usually fail to respond to conventional anticancer drugs and are therefore associated with a poor prognosis. To gain further insight into chemoresistance of human mast cells, the effect of etoposide, hydroxyurea, methotrexate, and cyclophosphamide on proliferation, viability, and apoptosis of the human leukemic mast cell line HMC-1 and primary cord blood-derived mast cells (CBMC) was evaluated. Proliferation was measured by microscopic counting of trypan blue-negative cells. To assess the number of viable and apoptotic cells, the uptake of PI and binding of annexin-V was investigated by flow cytometry. Immunoblotting was additionally performed to investigate the expression of bcl-2, bax, and bcl-xL after incubation with anticancer drugs. Whereas etoposide (0.01–1 μg per ml), hydroxyurea (1–100 μg per ml), and methotrexate (1–100 μg per ml), but not cyclophosphamide (1–100 μg per ml), were able to inhibit proliferation and viability and to induce apoptosis of HMC-1 cells in a dose- and time-dependent manner (24–72 h), CBMC were at first resistant to anticancer drug-induced apoptosis. However, preliminary results showed that after the addition of dexamethasone (0.4 μg per ml) or TRAIL (500 ng per ml), CBMC also exhibited apoptosis in response to anticancer drugs after 48 h. In HMC-1 cells, apoptosis following treatment with etoposide and methotrexate was associated with an increased expression of the pro-apoptotic protein bax and a decreased expression of antiapoptotic bcl-2 and bcl-xL. Thus, etoposide, hydroxyurea, and methotrexate are able to regulate proliferation and apoptosis of human mast cells *In vitro* and may therefore be further explored for the treatment of aggressive forms of mastocytosis.

019

The Stimulation of the Dermal and Epidermal Metabolisms: A New Antiaging Approach

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When the skin is young and sensitive to its environment, the epidermal cells are able to produce the proteins which are in charge of the cutaneous structure and organisation, as well as small peptides and growth factors which send out signals of synthesis or reparation to the surrounding cells. The regulation of this biosynthesis represents the specific molecular keys of a youthful skin and ensures the firness, elasticity and protective role of this young skin. As the skin becomes older, the cellular renewal slows down and there are more and more biosynthesis abnormalities which are responsible for the decrease of the endogeneous production of proteins and growth factors. Our reseach Laboratories have isolated and purified an oligosaccharide fraction rich in galacturonic acids from the flowery heads of Malva sylvestris. This extract is able to fight against the reduced metabolism of senescent and deficient epidermal and dermal cells and can biologically ''wake up'' certain metabolic activities. Therefore, via *in vitro* studies using monolayer cell cultures, our oligosaccharide fraction influences the epidermal cells' metabolism by on the one hand restoring the proliferation capacities of senescent human keratinocytes and on the other hand favorising keratinocyte synthesis such as the production of filaggrine, a structural protein of the corneocyte envelope or the synthesis of perlecan type proteoglycanes. In parallel, our molecular complex plays an important role in the activity of dermal cells by stimulating the fibroblasts proliferation capacity particularly in drastic culture conditions (culture medium deficient in nutriments and growth factors) and by increasing collagen synthesis. This effective cellular activity can be compared with a typical Retinoic acid response profile, recognised as the cutaneous antiage molecule reference. These results may prove to be an answer to the armful effects of aging on the skin and invite us to consider our oligosaccharide fraction as a potentiel candidate for antiaging program.

020

FXIII-Mediated Down-Regulation of the Urokinase-Type Plasminogen Activator (uPA) and Its Receptor (CD87) in Venous Ulcers

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Pattern of expression and state of activity of fibrinolytic factors after treatment of venous ulcers with the fibrin stabilizing factor XIII. The plasminogen activation system involving the urokinase-type plasminogen activator (uPA) and the urokinase-type plasminogen activator receptor (CD87) have been studied in tissue specimen on the mRNA- and protein expression by using reverse transcription followed by polymerase chain reaction, Western blot, immunohistochemistry and fibrin zymography after topical application of fibrin stabilizing factor XIII on venous ulcers. The fibrin stabilizing factor XIII displays accelerated wound healing, when applied on venous ulcers. Our results shows that local application of the fibrin stabilizing factor XIII reduces the mRNA- and protein expression of uPA and its receptor (CD87) as well as the functional fibrinolytic activity. These data strongly suggest, that wound healing in venous ulcers requires down-regulation of the fibrinolytic activity and characterizes the pharmacological effect of the fibrin stabilizing factor XIII on molecular level.

021

The Influence of Lysophosphatidic Acid on the Functions of Human Dendritic Cells

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Lysophosphatidic acid (LPA) is a bioactive lipid mediator generated by secretory phospholipase A_2, which accumulates in extracellular fluids of inflammatory sites. Here we studied the biological activity of LPA on human dendritic cells (DC), which are specialized antigen presenting cells characterized by their ability to migrate into target sites and secondary lymphoid organs in order to process antigens and activate naive T cells. We show that immature and mature DC express the mRNA for different LPA receptors such as EDG-2, EDG-4 and EDG-7. In immature DC, LPA stimulated pertussis toxin-sensitive Ca^{2+} increase, actin-polymerization and chemotaxis. During the maturation process, DC lost their ability to respond towards LPA with Ca^{2+} transients, actin polymerization and chemotaxis. However, LPA inhibited in a pertussis toxin-insensitive manner the secretion of interleukin 12 and tumor necrosis factor α as well as enhanced secretion of IL-10 from mature DC. Moreover, it increased the allostimulatory function of mature DC and favored the emergence of a Th2 immune response. In summary, our study implicates that LPA might regulate the trafficking, cytokine production, T cell activating functions of DC and ulimately favor. Th2 lymphocyte-dominated immunity.

022

HETE and F2-Isoprostanes in UV-Irradiated Human Skin and HaCaT-Cultures After Administration of Diclofenac and Acetylsalicylicacid Using Microdialysis Technique

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UV-irradiation of the human skin leads to induction of oxidative stress and inflammation mediated by reactive oxygen radicals, lipid peroxidation, liberation of arachidonic acid from membrane phospholipids and formation of prostaglandins and leucotrienes. Therefore, we investigated ''lipid mediators'', such as 8-epi-$PGF_{2\alpha}$, $9\alpha11\alpha$-$PGF_{2\alpha}$, HETEs and LTB_4 in the dermal interstitial fluid obtained *In vivo* by cutaneous microdialysis technique and *In vitro* from keratinocyte (HaCaT) cultures after UV-irradiation and application of diclofenac, a nonsteroidal anti-inflammatory drug. Defined areas on the volar forearm of 10 healthy volunteers were exposed to UVB irradiation (20–60 mJ per cm^2). After 24 h, microdialysis membranes were cutaneously inserted beneath the irriadiated area and diclofenac was administered topically. The probes were perfused with isotonic saline solution and microdialysate samples were collected at 20 min intervals over up to 6 h. Analysis of oxidized arachidonic acid derivatives using sensitive NICI-GC-MS showed enhanced amounts of 2-, 3-, 5-,12- and 15-HETE, LTB4, 8-epi-$PGF_{2\alpha}$ and $9\alpha11\alpha$-$PGF_{2\alpha}$ in the picomol range after UV irradiation, which were suppressed by topical diclofenac and acetylsalicylacid. Further investigations may show whether these new findings may also be relevant to validate therapeutical strategies for other inflammatory skin conditions.

023

Adaptation Response in Human Skin Barrier to a Hot and Dry Environment

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The epidermal permeability barrier, which resides in the *stratum corneum* (SC), is considered to be a biosensor that regulates epidermal responses to various exogenous insults. In order to investigate the effect of prolonged exposure to an environment with low relative humidity (RH) we compared the barrier strength and SC structure of panelists living in Arizona (USA, 27% RH in June) to panelists living in New York (USA) and Oevel (Belgium) (both 80% RH in July). We have previously reported that the SC of dry, xerotic skin showed lower levels of active proteases, e.g. the *stratum corneum* chymotryptic enzyme (SCCE), and a decrease in ratio of ceramides to proteins. Tape strippings from xerotic skin contained more protein, which has been correlated to increasing corneocyte density by image analysis. Skin chronically exposed to a hot, dry environment exhibited stronger skin barrier functions and lower basal transepidermal water loss. Fewer proteins were removed by tape stripping, suggesting less xerosis. RH may affect the rate of desquamation by modulating the *in situ* activity of desquamatory enzymes. The amount of active SCCE was higher in the skin of panelists exposed to low RH, suggesting that up-regulation of the amount of active proteases may be part of an adaptation response to ensure proper desquamation at low humidity. Calcium ions modulate barrier formation by inducing terminal differentiation, formation of the cornified envelope and regulating lipid secretion. Higher calcium levels were found in the upper SC of the Arizona panel, which might induce sustained lamellar body secretion. Taken together these data suggest that human skin can adapt to a low humidity environment by increasing epidermal barrier function and modulating desquamation. The distribution of calcium ions may constitute an internal signal for this adaptation response.

024

Bioconversion of Topical β-Carotene to Retinyl Esters in *Ex Vivo* Human Skin

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Background: The human epidermis contains endogenous retinoids (retinol, retinyl esters) and carotenoids (β-carotene). Several studies have shown that the enzymes involved in retinoid metabolism are present in human epidermis. There is still a controversy about the presence in the skin of the enzymes able to convert β-carotene into vitamin A (retinol). Andersson and coll. demonstrated recently that β-carotene can be converted into vitamin A in human cultured keratinocytes and melanocytes. Objective: We applied β-carotene and natural retinoids on the surface of *ex vivo* human skin to study their penetration into the epidermis and their bioconversion into vitamin A. Methods: Fresh surgically excised human abdominal skin was mounted in modified Franz perfusion chambers. Retinal, retinol, retinoic acid (0.05%, 2.5 mg per cm^2) or β-carotene (228 nmol per cm^2) were applied in the donor chamber; the receptor chamber was filled with a culture medium. The skin was incubated for 24 hours at 37°C, then epidermis was separated from dermis by heat (30 seconds at 56°C). High pressure liquid chromatography was used for the quantitative determinations of retinoids and carotenoids. Results: 24 hours following topical application with β-carotene, human skin was highly loaded with β-carotene (57 nmol per g), and epidermal retinyl ester concentration was increased to 2240 pmol per g, as compared to 124 pmol per g in untreated skin, whereas epidermal retinol was under the limit of detection of 10 pmol per g (untreated skin: 117 pmol per g). Topical retinal increased epidermal retinol and retinyl esters to 1635 and 353 pmol per g, respectively; after topical retinol, these values were 8311 and 697 pmol per g, respectively. Topical retinoic acid penetrated the epidermis (8124 pmol per g), and showed no metabolism. No retinoic acid was shown after topical retinal, retinol or β-carotene. Conclusion: Our results demonstraed that topical β-carotene is converted into retinol by human epidermis in an *ex vivo* model, and that retinol is then esterified with fatty acids. Since retinyl esters can be converted by the epidermis into the biologically active retinoic acids, topical β-carotene appears as a precursor of epidermal vitamin A.

025

Acute UVB Irradiation Does Not Modify the Penetration of Topical Retinoids in *Ex Vivo* Mouse Skin, But Alters their Metabolism

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In the present study, we assessed the effects of a single UVB exposure on the percutaneous absorption of retinal (RAL) and retinol (ROL), as well as their metabolism by the skin, in *ex vivo* mouse skin, using a tape-stripping method (Pirot *et al* PNAS 94:1562–1567 (1997)). Hairless mice were exposed to single UVB dose of 1 J per cm^2 under a flux of 0.28 mW per cm^2. One hour following UVB irradiation, the mice were sacrificed, and the skin was taken off and mounted onto Franz diffusion cells in which receptor compartment was filled with saline solution supplemented with PEG 400. *Ex vivo* retinoid metabolism and skin absorption. Donor compartment of Franz diffusion cell was filled with either 0.05% RAL or 0.05% ROL preparation (2 mg per cm^2). Receptor fluid was withdrawn at regular intervals on a period of time of 6 h. The epidermis was then removed by successive tape-stripping. After chemical extraction, RAL, ROL, retinoic acid (RA) and 13-*cis* RA (13cRA) content into tape strips, dermis and receptor fluid solutions were assayed by HPLC. No retinoid was detected in the receptor fluid solutions. The epidermal concentration profiles of ROL and RAL, following topical ROL and RAL, respectively, were not altered by a pre-exposure to UVB. In nonirradiated skin, epidermal ROL was the only retinoid found after topical ROL, whereas topical RAL produced sizeable amount of RA, 13cRA and ROL in the upper layers, respectively, 200.8, 172.8 and 33.4 nmol per g of epidermis. RAL applied to synthetic membranes showed no formation of RA or ROL. RAL applied on *ex vivo* skin one hour after UVB exposure produced less RA (58.5 nmol per g) and 13cRA (53.4 nmol per g), and no ROL. This indicates that a single UVB irradiation do not modify the penetration of topical retinoids in *ex vivo* mouse epidermis. However the capacity of the upper layers to metabolise retinoids has been significantly altered.

026

Involvement of Prostaglandins in Sebocytic Lipogenesis *In Vivo* and *In Vitro*: Indomethacin Augments the Production of Prostaglandin J_2 by Cyclooxygenase-Independent Pathways

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Sebaceous glands are important skin appendages and sebum excretion is considered to be associated with the functional maintenance of the surface of skin as a biological barrier. Sebaceous gland cells (sebocytes) differentiate and sequentially form intracellular lipid droplets. Recently, it has been reported that overexpression of cyclooxygenase-2 (COX-2) in epidermis of transgenic mouse skin has caused hyperplasia of sebaceous glands and augmented the accumulation of lipid droplets. In the present study, to clarify the involvement of prostaglandins (PGs) in sebocytic lipogenesis, we examined the effect of PGs and a COX inhibitor, indomethacin on the formation of lipid droplets and the function of sebaceous glands of hamster *in vitro* and *in vivo*. Various PGs such as PGE_2, PGF_{2a} and PGJ_2 were detected in the culture medium of hamster sebocytes and the levels of PGF_{2a} and PGJ_2 were increased along with the insulin-induced augmentation of lipogenesis. In addition, exogenous $PGF_{2\alpha}$ and PGJ_2 augmented the accumulation of lipid droplets, which mainly consisted of triglycerides. When the sebocytes were treated with indomethacin, the production of $PGF_{2\alpha}$ and PGE_2 decreased, but the level of PGJ_2 in the medium increased. Furthermore, the augmented formation of intracellular lipid droplets was observed in the indomethacin-treated sebocytes. When indomethacin was applied to auricles of 5-week-old male golden hamsters, the development of sebaceous glands was also observed. Therefore, these results suggest that PGs such as PGJ_2 and $PGF_{2\alpha}$ may play roles as endogenous regulators for stimulating lipogenesis in hamster sebaceous glands. Moreover, we suggest novel evidence that a by-pass to synthesize PGJ_2 from arachidonic acid may be evoked by intercepting the COX pathways in hamster sebocytes.

027

Intercellular Lipid Lamellar Structure in Hairless Mouse Stratum Corneum at Various Water Contents

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Lamellar structures of intercellular lipids in stratum corneum of hairless mouse were investigated at various water contents by small-angle X-ray diffraction. At water content 21 wt%, we observed long and short lamellar structures with repeat distances of 13.6 nm and 6.0 nm at room temperature, respectively. The spacing of the long lamellar structure is almost constant over the water content from 0 wt% to 80 wt%. This result is consistent with the previously reported one. For the short lamellar structure, we found that with increasing the water content the lamellar spacing becomes large, that is, from 12 wt% to 50 wt% the short lamellar spacing increases from 5.8 nm to 6.6 nm. In addition to the previously reported result that at the water content of about 20 wt% the X-ray diffraction peak for the long lamellar structure becomes sharp, we found that this is also the case for the short lamellar structure. Below the water content of about 12 wt% the X-ray diffraction peak for the short lamellar structure disappers. On the other hand, above the water content of about 50 wt%, it becomes weak and finally merges into the second-order diffraction peak for the long lamellar structure. From the behavior of the lamellar spacings and sharpness of diffraction peak as a function of the water content, it is speculated that an anhydrous long lamellar structure correlates with double short lamellar spacing with water layer. It is likely that the long and short lamellar structures of the intercellular lipid play an important role in the regulation of water stored in stratum corneum.

028

Reduced Activity of Ceramide Generating Acid Sphingomyelinase in Atopic Dermatitis

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A defect in permeability barrier function, leading to the penetration of environmental allergens into the skin is crucially involved in the pathogenesis of atopic dermatitis. Allergens induce immunological reactions leading to inflammation. Previous studies showed that the decreased level of stratum corneum ceramides may be the cause for the defect in permeability barrier function in atopic dermatitis. We recently described that acid sphingomyelinase (A-SMase) generates specific ceramides for permeability barrier function. A-SMase is localized predominantly in the endosomal epidermal lamellar bodies of the upper stratum spinosum/stratum granulosum, from which the lipids are delivered to the intercellular lipid bilayers of the stratum corneum. The aim of the present study was to determine whether A-SMase activity is impaired in atopic dermatitis. We performed 5 mm punch biopsies from lesional and nonlesional skin in patients with atopic dermatitis and got skin samples from healthy controls. The epidermis was separated from the dermis and specific enzyme assay was performed. We found a significant decrease in epidermal A-SMase activity in lesional (–48%) and nonlesional skin (–73%) of patients with atopic dermatitis compared to healthy controls. In contrast, preliminary studies in allergic patch test reactions revealed an increase in SMase activity. Recently, it was shown that epidermal A-SMase specifically generates stratum corneum ceramides 2 and 5 subtypes. Ceramide 2 is quantitatively the most important ceramide in the epidermis. Therefore, it is most likely that the specifically reduced A-SMase activity in atopic dermatitis, as we found here, is responsible for the decrease in stratum corneum ceramide and the reduced barrier function. In summary, we found a reduced epidermal A-SMase activity correlating with the reduced ceramide content known in atopic dermatitis.

029 [Oral 004]

Injection of a Soluble Form of Ectodysplasin A (EDA) Stably Reverts the Murine Equivalent of X-Linked Hypohydrotic Ectodermal Dyslasia (XL-HED)

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Hypohidrotic Ectodermal Dysplasias are characterized by defects in the development of structure of ectodermal origin such as hair, teeth and sweat glands. Most forms of the disease have been shown to implicate defects of the signaling pathway of the TNF family ligand Ectodysplasin A (EDA). Absence or deficient expression of EDA itself give rise to X-Linked HED type I, the most common form of the disease. In mice, absence of EDA results in the Tabby phenotype, which resembles human HED in many aspects, thus providing an excellent animal model for this condition. Based on functional analysis of point mutations found in patients, we could show that EDA activity depends on ligand shedding from the membrane as well as ligand aggregation by its collagen domain. This prompted us to engineer a soluble and aggregated form of EDA that can cross the placental barrier and reach the developing embryo. This treatment stably reverts most of the Tabby defects and represents one of the rare examples where a genetic disorder can be treated by administration of a recombinant protein. Our results indicate that this recombinant EDA holds interesting promises for both therapy and fundamental research.

030 [Oral 054]

Interactions Between HIV and Dendritic Cells: Turning the Pathogen Into a DC-Specific Vector

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Since new HIV infections occur primarily through sexual transmission, small amounts of virus have to cross the mucosal barrier and reach replication-competent sites. Evidence has accumulated that a specific subtype of cells, called Langerhans/dendritic (DC) cells, plays a crucial role in the early events of entry of the virus in the host. HIV can avoid digestion by the DC and uses their migratory properties in order to gain access to the lymph nodes, where maximal viral replication occurs. We provide evidence of how HIV escapes degradation and processing in DC and replicates actively in DC-T cell clusters, which contributes to HIV pathogenesis. However, HIV not only prevails as a dangerous parasite, but can be modified into a useful vector for DC-based therapies. Attenuated variants of HIV/SIV are promising tools to genetically modify DC, usually resistant to physical transfection methods. Lentiviral vectors efficiently transduce human CD34+ stem cell derived DC, monocyte derived DC, as well as murine bone marrow derived DC, with transduction rates up to 90%. Furthermore, we will demonstrate that targeting of dendritic cells by LCMV antigen-encoding lentiviral vectors permits antigen processing and MHC class I dependant presentation. Our results point to lentivectors as promising candidates for DC-based immunotherapy purposes due to high transduction ability, targeting properties via DC-specific promoters and capacity of inducing Ag-specific immune responses. Together, these studies illustrate the complex interactions between dendritic cells and HIV and their potential implications for immunotherapy strategies.

031 [Oral 055]

A Functional Knockout of Human Keratin 10: A New Clinical Entity of Bullous Congenital Ichthyosis

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Epidermolytic hyperkeratosis (EHK) is generally inherited as an autosomal dominant trait and caused by mutations in keratin 1 and 10 (K1 AND K10). In the here described family two children from consanguineous clinically unaffected parents exhibited typical clinical features of severe EHK such as generalized exfoliative scaling erythroderma. Palms and soles were not affected. The diagnosis was confirmed by electron microscopy demonstrating abundant keratin tonofilament aggregates in the suprabasal epidermal layers in both affected children. Surprisingly these keratin clumps did not show filamentous, striped aspects as in autosomal dominant EHK but were completely amorphous. Perinuclear shells of keratin clumps were absent. Sequence analysis of PCR amplified DNA fragments from both affected children using specific primers for keratin genes 1 and 10 (KRT1 AND KRT10) revealed a homozygous CAA to TAA base pair exchange at position 1276 in exon 6 of the KRT10. Both parents were heterozygous carriers of this mutation. The mutation results in a premature stop codon (Q426X) 25 amino acid residues before the end of the 2B rod domain of K10. Immunohistochemistry applying a specific polyclonal antibody against the C-terminus of K10 failed to detect K10 in keratin extracts of cultured keratinocytes of the affected children. Semiquantitative rT-PCR showed almost complete reduction of specific KRT10 mRNA levels in cultured keratinocytes. These results suggest that the mutant KRT10 mRNA is mostly degraded due to nonsense mediated RNA decay and is not translated in a stable truncated K10. In the heterozygous parents this defect is obviously compensated by the remaining unaffected KRT10 allele. The absence of K10 in the epidermis of our patients resulted in a disease phenotype resembling autosomal dominant EHK where the expression of a mutated K10 exerts a dominant negative effect on keratin intermediate filament formation. This observation is in contrast to a recently established KRT10 knockout mouse model that developed an intact epidermis without signs of increased skin fragility. In these mice the lack of K10 seems to be compensated by decreased proteolysis of the basal keratins K5 and K14 and there persistence into suprabasal epidermal layers. Current investigations aim to establish the molecular correlate for this pronounced differences between the mouse model and the human KRT10 knockout.

032 [Oral 056]

Tissue and Cell Characterization of Lympho-Epithelial Kazal-Type Related Inhibitor (LEKTI) Provides Evidence for Defective Expression in Netherton Syndrome

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LEKTI is a predicted 120 kDa serine protease inhibitor organised in 15 potential inhibitory Kazal-type domains (D1-D15). It is produced as the precursor of at least 3 proteolytic fragments: D1 (6.5 kDa), D6 (7.5 kDa) and D8-D11 (30 kDa) among which D6 was demonstrated to inhibit trypsin activity *In vitro*. LEKTI is encoded by SPINK5 which we recently identified as the defective gene in Netherton Syndrome (NS), a severe autosomal recessive ichthyosis characterised by congenital erythroderma, a specific hair-shaft abnormality and atopic manifestations. In order to study LEKTI tissue and cell expression, we developed rabbit polyclonal antibodies raised against recombinant N-terminal (D1-D6) and C-terminal (D13-D15) parts of the molecule produced as GST-fusion proteins in *E. coli*. Immunohistochemical analysis showed strong and specific labelling in the granular layer of the epidermis, in the hair follicle and sebaceous gland epithelium. Localised expression of LEKTI was found in epithelial cells of the thymus, suggestive of Hassal bodies. Oral mucosa, tonsil and genital epithelia also showed strong expression of the protein. In contrast, LEKTI expression was not detectable in skin sections of NS patients. Lysates from sub- and postconfluent normal vs. NS human primary keratinocyte (HPK) cultures were analysed by Western blotting. The presence of a 120-kDa protein, consistent with the expected relative molecular weight of LEKTI, was detected in normal postconfluent HPK and not in NS HPK. Western blot analysis of conditioned media from transfected COS cells over-expressing the C-terminal myc-tagged protein confirmed the expression of the 120 kDa LEKTI precursor and allowed the identification of a 75-kDa C-terminal proteolytic fragment predicted to correspond to polypeptide D7-D15. Additional fragments of smaller size were also detected with antibodies to D1-D6 and may correspond to N-terminal proteolytic products. The antisera against LEKTI which we have developed represent powerful tools for rapid diagnosis of NS. They will also prove useful for further analysis of LEKTI expression and identification of its interacting molecules.

033 [Oral 059]

Spectrum of Mutations in the ECM1 Gene in Lipoid Proteinosis

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We recently identified homozygous nonsense mutations in the extracellular matrix protein 1 gene, *ECM1*, in the autosomal recessive disorder lipoid proteinosis (Hamada T *et al* Hum Mol Genet 2002; 11: 833–40). In the present study, we sequenced *ECM1* in 4 further patients to extend genotype-phenotype correlation and to add to the mutation database. The patients comprised two Thai brothers (aged 38 and 19 years) with severe laryngeal hoarseness and scarring; the third patient was an unrelated 30-year-old Thai woman with very mild hoarseness and a few eyelid papules; the fourth patient was a 23-year-old Indian man with moderately severe skin changes, hoarseness and temporal lobe calcification. Sequencing of genomic DNA disclosed novel homozygous single base-pair deletions: 507delT in the Thai brothers, 243delG in the other Thai patient and 785delA in the Indian patient. The latter mutation occurs in the normally differentially spliced exon 7 and is predicted to ablate the ECM1a isoform but not the shorter ECM1b transcript. Conversely, the mutations in the Thai patients (exons 4 and 6) are expected to completely ablate both ECM1a and ECM1b. To explain phenotypic diversity in the Thai patients, RT-PCR using cDNA from cultured keratinocytes provided evidence for partial rescue of the mutant transcripts with restoration of the open reading frame through in-frame skipping of exons 3 and 4 in the patient with mild disease, but no such changes in the more severely affected brothers. In summary, 9 different homozygous *ECM1* mutations in lipoid proteinosis have now been reported, most of which occur in exon 7. However, lipoid proteinosis is clinically heterogeneous and accurate clinicopathological correlation also requires RNA and protein analysis.

034 [Oral 060]

A Canine Model for Somatic Gene Therapy of Junctional Epidermolysis Bullosa

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Our study is aimed at the perfection of somatic gene therapy of junctional epidermolysis bullosa (JEB), a heterogeneous group of skin blistering diseases associated with genetic mutations in the genes for the adhesion ligand laminin 5. Since the full phenotypic reversion of human JEB keratinocytes has been achieved *ex vivo*, we have established a spontaneous animal model of JEB suitable for the preclinical validation of the gene therapy approach. Genetic analysis of German Shorthaired Pointers with mild JEB demonstrated that the affected animals carry the homozygous missense mutation 1514C-to-T in the laminin-5 a3 chain. Transfer of the wild-type canine a3 cDNA (5,1 kb) into the dog JEB keratinocytes using a retroviral MMLV-based vector enhanced the secretion of laminin 5 in the extracellular matrix and restored the adhesion and proliferation capacity of the transduced cells. The clonogenic potential of the reverted keratinocytes was also fully recovered, which demonstrates that the dominant positive effect of the wild-type laminin a3 chain results in the assembly of functional laminin-5 molecules. The reverted dog keratinocytes have been used to reconstruct autologous transplantable epithelia that have been grafted onto three JEB dogs. This approach will provide information on the tolerance that immune competent hosts have for genetically engineered JEB epithelia expressing recombinant laminin 5 molecules and will provide the proof of efficacy for the genetic therapy of inherited skin blistering diseases.

035

Uptake, Trafficking and Expression of Naked Plasmid DNA in Human Keratinocytes

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The skin is an interesting organ for human gene therapy due to its easy accessibility, immunologic potential and synthesis capabilities. Therefore, it is important to understand the uptake and transport mechanisms of DNA in keratinocytes with the goal to optimize therapeutic approaches in skin gene therapy. In previous work we have shown that naked plasmid DNA is taken up and expressed by human keratinocytes upon *in vivo* intradermal injection. Yet, the mechanism by which plasmids surmount the cell membrane and enter keratinocytes as well as their intracellular trafficking still remain unknown. We measured uptake of FITC-labeled plasmid by FACS analysis after 24 h incubation detecting 5–10% internalization in a dose- and time-dependent manner. Furthermore, we demonstrate that cycloheximide treatment inhibited uptake by $>90\%$, suggesting a protein-mediated uptake. Next, we attempted to inhibit selected alternative internalization pathways such as macropinocytosis (by dimethylamylorid), clathrin-coated pits (by chlorpromazine) and caveolae (by nystatin/filipin). Most prominently, blocking macropinocytosis reduced the uptake by $>85\%$, while chlorpromazine and nystatin had no inhibitory effect. Finally, colocalization studies by confocal laser microscopy revealed a time-dependent accumulation of plasmid DNA in endosomes (FITC-dextran) and lysosomes (LysoTracker Green®). To detect potential DNA receptors on the keratinocyte surface, two DNA-binding proteins, ezrin and moesin, were identified in 2-D South-Western blots and MALDI-mass spectrometry using linearized calf thymus and λ phage DNA. Although these actin-membrane linker proteins are localized intracellularly, they are functionally associated with a number of transmembrane receptors such as the EGF- or ICAM-1-receptor. Taken together naked plasmid DNA seems to enter human keratinocytes through different pathways. Compared with linear calf thymus DNA, plasmid DNA seems to be less abundant in endosomes and lysosomes, making it particularly suitable for skin gene therapy.

036

Dyschromatosis Symmetric Hereditaria: Analyses of Clinical and Genetic Features

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Objective: To study clinical and genetic features of Dyschromatosis Symmetric Hereditaria (DSH) in Chinese. Methods we studied the clinical and genetic features of DSH by reviewing 8 families reported in China since 1980. Results: The pattern of inheritance was autosomal dominant. The patients showed a mixed of hyperpigmented and hypopigmented macules symmetrically distributed on the extremities ,which are typical DSH. A few of DSH patients showed freckle-like pigmented macules on the face or spreaded on the trunk. DSH occured in infancy or childhood. There was different expressity in different patients of the same family. The patients with DSH in Family IV were also affected by Ichthyosis Vulgaris. Patients with DSH in other families were not affected by other diseases. Family V showed a irregular dominance. Conclusions: DSH generally shows an autosomal dominant pattern of inheritance with high penetrance and it is not a rare disease in Chinese. The typical DSH is characterized by a mixed of hyperpigmented and hypopigmented macules on the extremities. There are differences in the expressity of patients.

037

Collagen Type VII (COL7A1) Mutation Survey and Genotype-Phenotype Correlation in Italian Patients Affected by Dystrophic Epidermolysis Bullosa

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Dystrophic epidermolysis bullosa (DEB) is a rare skin disorder showing clinical heterogeneity and transmitted either in dominant (DDEB) or recessive (RDEB) mode. All variants of DEB have been associated to mutations in type VII collagen gene (COL7A1). We report the survey of COL7A1 mutations in 51 Italian DEB patients, 27 affected by Hallopeau-Siemens RDEB (HS-RDEB), 19 by non HS-RDEB, 2 by DDEB, 2 by pretibial DEB (Pt-DEB) and 1 by inversa RDEB (I-RDEB). 42 mutations were identified, 18 of which are novel. Mutation consequences were analyzed at the mRNA and protein level and genotype-phenotype correlation drawn. The recessive inheritance of 2 cases of Pt-DEB was also established. In RDEB patients, 6 recurrent mutations were identified: 7344G→A, 425A→G, 8441–14del21, 4783–1G→A, 497insA and G1664A, the last three being found only in Italian patients. Haplotype analysis and patient geographic origin attested the propagation of ancestral mutated alleles within the Italian population. Altogether recurrent mutations cover about 43% of RDEB alleles in Italian patients and therefore new DEB patients should first be screened for the presence of these mutations.

038

Expression of the Psoriasis Candidate Gene, HCR, Located in the PSORS1, is Influenced by Proliferation and Cytokines

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The genetic susceptibility to psoriasis is most clearly linked and associated to the PSORS1 region near HLA-C in chromosome 6p. We are studying the genes located in this region as candidates for the biological effect with a role in the pathogenesis of psoriasis. A strong candidate gene, HCR (for α-helical coiled-coil rod protein), is located 115 kb away from HLA-C. Our initial analyses showed that the HCR gene is highly polymorphic and that it has specific coding SNPs that associate strongly with psoriasis. In order to refine the possible role of HCR in psoriasis, we undertook to study (1) whether the same alleles associate with psoriasis in different populations; (2) how strong the associations are in comparison with other genes in the PSORS1 region; (3) whether HCR expression changes in psoriasis and other differential diagnostic skin disorders; and (4) whether cytokines and regulators of keratinocyte function affect HCR expression. In addition, we are in the process of developing cell culture models to study HCR function. Our results have shown that the same set of coding SNPs form a specific susceptibility allele that is a genetic risk factor for psoriasis in at least 7 distinct populations from Europe and Asia. The genetic association parallels that observed for HLA-Cw6 and surpasses CDSN (corneodesmosin) associations. No other known genes in the region have allele frequencies that would offer stronger associations. HCR expression is qualitatively changed in psoriatic lesions as compared to normal skin with an altered profile in rete ridges. HCR is also expressed in certain epithelial cancers suggesting that proliferation or cell cycle can influence its expression. Finally, biologically active cytokines regulate HCR expression levels. We conclude that HCR is a good candidate for an effector gene in the PSORS1 region.

039

Identification and Characterization of an RNA Gene Related to Psoriasis Susceptibility

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In order to identify transcriptional level differences between psoriatic noninvolved and healthy epidermis contributing to psoriasis susceptibility, differential display (DD) experiments were carried out. Beside several genes with already well characterized functions (fibronectin, RAB10), differentially regulated cDNAs with yet unknown functions were identified. One of these cDNAs showed 100% homology to a transcribed gene sequenced from a human 10-week-old foetal cDNA library, with GeneBank accession number AK022045. The gene is localized on chromosome 10 and *in silico* translation resulted in no translatable protein product on its nucleotide sequence. The 5′ end of the gene harbours an Sq subtype *Alu* element and two conserved RNA polymerase III promoter elements but no small nuclear RNA conservative elements. These characteristics reveal a high level similarity to the BC200 RNA gene that encodes a neural small cytoplasmic RNA and shows abnormal expression in non-neuronal tumors. Reverse-Southern and RT-PCR experiments on numerous independent samples proved that AK022045 is up-regulated in the noninvolved epidermis of psoriatic patients compared to healthy epidermis and shows even higher expression in the psoriatic plaques. *In vitro* expression studies on HaCaT cells and keratinocytes demonstrated the expression of AK022045 in both cell types and its up-regulation under certain stress conditions (contact inhibition, serum-starvation, UV-B irradiation, heat shock, LPS combined with inflammatory cytokines). Cultured melanocytes, an immortalized melanocyte cell line and a melanoma cell line also expressed the AK022045 RNA. The level of expression was significantly higher in the immortalized cell line and in the melanoma cell line compared to normal cultured melanocytes.

040

Role of the Autoimmune Regulator (AIRE) Gene in Alopecia Areata: Strong Association of a Potentially Functional AIRE Polymorphism with Alopecia Universalis

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Alopecia areata (AA) is a chronic inflammatory disease characterised by patchy hair loss with T cell infiltration of hair follicles. The lifetime risk of AA is approximately 1% in the general population, but this is increased to 30% or more in the autoimmune polyendocrinopathy candidiasis ectodermal dysplasia syndrome (APECED), a recessive disorder due to mutation of the autoimmune regulator (AIRE) gene on chromosome 21q22.3. We have therefore studied the possible involvement of *AIRE* in the pathogenesis of AA. On screening the *AIRE* coding sequence, we identified 22 variants in the AIRE gene sequence. Two variants at positions G961C and T1029C gave amino acid changes S278R and V301A located in the DNA-binding segment (SAND) and PHD1 zinc finger motif, respectively. We have developed genotyping assays for both G961C and T1029C variants. The *AIRE* T1029C variant was not informative and the frequency of the rare allele (961C) of the *AIRE* G961C variant was 0.08 in our control group. We developed differential double enzyme assays to discriminate the *AIRE* G961C alleles and genotyped 202 AA patients and 175 matched Caucasian controls. There was a significant increase in the frequency of the 961C allele in AA patients overall to 0.13. This increase was higher in severe disease (alopecia universalis) 0.21. We found no association between the *AIRE* G961C variant and mild (patchy) AA or alopecia totalis. However, the *AIRE* 961C allele presents a strong risk factor (more than three times) for development of the most severe form of AA [(OR) = 3.27 (1.56,6.88) $p = 0.0012$]. Early age of onset (< 30 year) is also a risk factor for disease [OR 2.22 (1.22,4.03) $p = 0.0082$]. The amino acid change from serine to arginine in the SAND domain of Aire protein may have a significant effect on Aire DNA-binding activity. Moreover, our results could provide a rational explanation of the unusually high frenquency of AA in APECED patients which also supports the concept of AA as an autoimmune disease.

041

Efficient Transgene Expression in Skin Equivalent Model Using Replication-Deficient Adenovirus Vector System

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It has been reported that skin equivalent model might be an effective means to treat genetic skin diseases such as epidermolysis bullosa, which can be treated by skin gene therapy in future. For *ex vivo* gene therapy, we investigated gene transfer into skin equivalent model. The adenovirus vector system is a highly effective tool of expressing foreign genes in various tissues. Therefore, we examined whether the adenovirus vector system could introduce foreign gene into skin equivalent model. We constructed Cre-loxP adenovirus vector carrying enhanced green fluorescence protein (EGFP) gene. First we examined EGFP expression in monolayer keratinocytes by coinfection of adenovirus carrying cre-recombinase and loxP-EGFP. EGFP was detected 6 h after infection in almost all of the cells, and its expression was observed in a time and dose dependent manner until 36 h. Next we examined the gene expression in skin equivalent model. When keratinocytes were infected with the adenovirus vector before seeding on collagen gels, EGFP expression was limited to the cornified cell layer. So, we changed the infection method. Skin equivalent was produced by air exposure, followed by direct injection between epidermis and dermis. Using this approach, EGFP was expressed prominently in the basal and suprabasal layers confirmed by confocal laser microscopy and graft model. These results demonstrate that the Cre-loxP adenovirus vector system is a useful vector for exogenous gene expression in skin equivalent model as well as in monolayer keratinocytes, and leads to an *ex vivo* gene therapy of skin diseases.

042

A Homozygous In-Frame Deletion in the Largest Collagenous Domain Decreases the Thermal Stability of Collagen XVII

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Collagen XVII is a transmembrane hemidesmosomal component which is associated with epidermal detachment in junctional epidermolysis bullosa (JEB). Most COL17A1 mutations described lead to a premature termination codon and cause absence of collagen XVII in skin. Here we analyzed the biological consequences of a COL17A1 in-frame deletion by studying the stability and integrin binding of wild-type and mutated collagen XVII domains. The mutation occurred in a newborn patient with JEB who was homozygous for the splice site mutation 2441–2 A to G. It results in an in-frame skipping of the entire exon 32 and causes a deletion of 9 amino acids in the largest collagenous domain of collagen XVII. Ultrastructural and immunofluoresence investigations revealed absence of collagen XVII and junctional blistering with intact keratinocytes in blister roof and normal lamina densa and anchoring fibrils in blister base. Site-directed mutagenesis was used to generate the deletion in an expression vector coding for the largest collagenous domain, Col15 and the mutated collagen was expressed in an eukaryotic episomal expression system. Trypsin digestions as probes for protein folding indicated that the stability of the mutated Col15 fragment was significantly reduced. The midpoint of the helix-to-coil transition, Tm, was 7°C lower than that of wild-type Col15 fragment. In cell spreading assays, HaCaT cells migrated similarly on cell culture wells precoated with wild-type or mutated Col15. Thus this deletion causes abnormal triple-helix folding and susceptibility to unspecific proteolysis of the ectodomain but has no effect on keratinocyte binding of collagen XVII.

043

Recessive Palmoplantar Keratoderma: Mapping and Candidate Gene Exclusion

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In the northern part of Sweden two types of hereditary nonepidermolytic palmoplantar keratodermas (PPKs) are present, one with autosomal dominant mode of inheritance (PPK type Bothnia) and one with autosomal recessive mode of inheritance (rPPK). PPK type Bothnia is the predominant disorder with a reported prevalence of 0.3–0.5%, whereas the prevalence of rPPK is about 2.7×10^{-5}. The phenotype of rPPK includes a sharply demarcated waxy diffuse palmoplantar hyperkeratosis usually accompanied by hyperkeratosis on dorsal aspects of fingers and toes. Additional features of the rPPK phenotype are dermatophyte infections and hyperhidrosis of palmoplantar skin, conical tapering of fingertips and maceration between toes. There are no associated ectodermal defects. We have collected DNA from the 13 known rPPK patients in Sweden and their unaffected relatives and performed a genome wide search for the disease locus. Linkage was found to microsatellite markers on chromosome 8qter, with a maximum LOD score of 5.64. Within the region all affected individuals shared a common haplotype, indicating a founder effect. The rPPK region on is approximately 2.3 Mb accordning to the USCS map and contains about 35 genes. The genodermatose Mal de Maleda has a similar phenotype to rPPK and maps to the same region. A recent report concludes that Mal de Maleda is caused by mutations in the ARS gene. We therefore analysed both intron and exon sequence of the ARS gene in our family material using denaturing-high-performance-chromatography (DHPLC). No polymorphims segregating with rPPK was found, leading to exclusion of ARS as a candidate for rPPK. Coding regions of 5 additional candidate genes have been analysed, however, no polymorphism segregating with rPPK has been detected so far.

044

Real Time Quantitative RT-PCR: A New Approach for Studying Expression of Markers for Ageing in Human Skin

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Skin aging can be divided into two simultaneous processes: intrinsic and extrinsic ageing due to environmental factors as chronic sun exposure (photoaging). Several studies were focused on molecular dermis features in photo-aged skin. Very little is known about epidermal markers and structural modifications due to ageing has been reported in basal cell keratinocytes. To investigate epidermal markers of the photo-ageing process for the face skin, we studied in wrinkled sun-exposed and in nonexposed control skin, transcripts coding for β1A integrin, a marker of basal keratinocytes and for involucrin, a marker of differenciation for keratinocytes. We developed a real-time quantitative RT-PCR assays to quantify these two transcripts in human skin biopsies. The RT-PCR were performed on isolated epidermis in conditions to obtain a detection level of 50 copies and an efficacy up to 90% with standard curves and samples. These two transcripts were detected and quantified in all biopsies studied. Our data showed that in 8 out of 10 patients, β1A-integrin transcripts were decreased in sun-exposed skin compared to control biopsies. For involucrin, variation of transcript between sun-exposed and control skin is proper to each patient. These data indicate that at mRNA level, β1A integrin is a marker of the photoaging process and could be considered for studying the effect of potential active compounds.

045

A Missense Mutation in CDH3, Encoding P-Cadherin, Causes Hypotrichosis with Juvenile Macular Dystrophy

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Hypotrichosis with juvenile macular dystrophy (HJMD) is a rare autosomal recessive disorder characterized by early hair loss heralding the occurrence of severe degenerative changes affecting the retinal macula and culminating in blindness during the second to third decade of life. We recently reported a frameshift mutation in the *CDH3* gene encoding p-cadherin, as the proximal cause of HJMD in 4 families. We identified an additional consanguineous family affected with HJMD and comprising 4 affected individuals. Light and scanning electron microscopy revealed morphological hair abnormalities consistent with pili torti in all patients. Fundus examination disclosed in all patients marked degenerative changes in the macular pigment epithelium. Electrophysiological studies were diagnostic of severe retinal dysfunction. Using haplotype analysis, we found out that all affected family members were homozygous for a polymorphic marker located in the vicinity of the *CDH3* gene. We establish the entire coding sequence of *CDH3* in a patient and identified a missense mutation in this gene resulting in a substitution at position 503 of P-cadherin amino acid sequence (R503H). The mutation was found in an homozygous state in all affected individuals, in an heterozygous state in obligatory carriers and in none of 83 healthy unrelated individuals. The mutation was shown to affect a highly conserved residue, considered to be involved in Ca^{+2} binding. This is the first missense mutation reported in CDH3 and the second mutation found to underlie HJMD. These findings establish mutations in CDH3 as the cause of HJMD and expand our understanding of the pathophysiology of this fascinating disorder.

046

Novel Mutation of Keratin 9 in a Hungarian Pedigree with Epidermolytic Palmoplantar Keratoderma

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Epidermolytic palmoplantar keratoderma (EPPK, MIM#144200) is an autosomal dominant disorder with hyperkeratosis confined to the palms and soles. It is characterized histologically by cytolysis within the stratum spinosum and granulosum. Mutations in the keratin 9 gene (KRT 9) encoding this type I keratin, expressed exclusively in the suprabasal keratinocytes of the palmoplantar epidermis, have previously been demonstrated in this disorder. We studied a large Hungarian pedigree presenting with EPPK and reviewed the literature. The diagnosis was confirmed by skin ultrastructural studies. A novel heterozygous missense mutation, 545 A→T, N160I in exon 1 of KRT 9, was detected. This new mutation located in the helix initiation motif at the start of the central coiled-coil rod domain of keratin 9. These findings provide further evidence for mutational heterogeneity in EPPK and underline the functional importance of the 1 A rod domain segment in the structural integrity of keratin intermediate filaments.

047

Mutation Analysis of the ATP2A2 Gene in Five Hungarian Patients with Darier's Disease

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Mutations in the ATP2A2 gene, encoding SERCA2, a P-type ATPase had been identified as the cause of Darier's disease (DD), an autosomal dominantly inherited skin disease. Mutation analysis in five Hungarian DD patients had been performed, to get more information about phenotype-genopyte relations. All of them had moderate to severe skin symptoms. PCR amplification of the entire coding region of ATP2A2 was performed. Mutation detection strategies included heteroduplex scanning by conformation-sensitive gel electrophoresis and direct nucleotid sequencing. We found distinct, heterozygous mutations (2 missense, 1 nonsense, 1 deletion and 1 insertion), 3 of which was novel. In a 31-year-old DD woman with learning difficulties we disclosed a previously described missense mutation (D702N) in exon 15, however, in that other patient no neuropsychiatric symptoms had been indicated. A 44-year-old mentally mildly handicapped DD woman had a T insertion at nucleotid 559 in exon 7 of the ATP2A2 gene, which resulted in a premature stop codon at codon 192. A woman, whose skin symptoms developed unusually late, at the age 50, had a T deletion (1320delT) in exon 11 resulting in a premature stop codon at codon 448. Our most severe case had a known missense mutation N39T, resulting in a nonconservative amino acid change at the upstream stalk region. A further new nonsense mutation (C909Z) was detected in the M8 transmembrane domain. International comparative genotype-phenotype analyses can be only performed from exact clinical databases.

048

Fluconazole Down-Regulates Metallothionein Gene in Dermatophyte (*M. canis*)

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Azole antifungals are widely used to treat infections with dermatophyte fungi. Whereas it is commonly accepted that this class of drugs interferes with fungal sterol synthesis, little is known about their potential other biological effects. To investigate *M. canis* genes regulated by fluconazole we cultured *M. canis* in the present of 50 μg fluconazole and subjected the RNA to differential display analysis. Differently expressed genes were isolated and sequenced. Here we demonstrate that fluconazole is able to down-regulate the baseline as well as copper-induced expression of Metallothionein by *M. canis*. Since this effect occurred within 30 min after exposure of the fungus to fluconazole, it is unlikely that it represents a secondary effect of cell wall destabilisation due to impaired sterol synthesis. Our additional demonstration that fluconazole enhances copper toxicity for *M. canis* suggest that inhibition of metallothionein expression by fluconazole is biologically relevant and may represent an important mode of the antifungal action of this drug. Therefore our data indicate that antifungal effects of azoles derivatives might not only be due to interference with cell wall synthesis but may affect other biological circuits within the fungal cells.

049

Targeted Inhibition of Human Collagenase-3 (MMP-13) Expression by Antisense Ribozyme Inhibits Squamous Cell Carcinoma Growth *In Vivo*

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Squamous cell carcinomas (SCCs) of the head and neck are characterized by high tendency to invade locally and metastasize. Collagenase-3 (matrix metalloproteinase-13, MMP-13) is expressed by tumor cells of SCCs and it is associated with high invasion capacity. To specifically examine the role of MMP-13 in the growth and invasion of SCC, we constructed a hammerhead ribozyme specifically targeted against human MMP-13 mRNA. The MMP-13 antisense ribozyme effectively cleaved MMP-13 transcripts *In vitro*. We also constructed a recombinant adenovirus, RAdMMP-13ASRz, to express MMP-13 antisense hammerhead ribozyme in living cells. Adenoviral delivery of MMP-13 antisense ribozyme into cutaneous SCC cells resulted in potent and specific inhibition in production of proMMP-13 and suppressed invasion of SCC cells through Matrigel by 80%. TUNEL positive cells were detected 72 h after the adenoviral delivery of MMP-13 antisense ribozyme into SCC cells, and Hoechst staining of SCC cells showed condensated nuclei within 96 h. SCC tumors established in SCID mice were injected with RAdMMP-13ASRz intratumorally 2–3 times a week (10^9pfu per dose) for three weeks. *In vivo* injection of RAdMMP-13ASRz into SCC tumors inhibited tumor growth by 50–75%. The gelatinolytic activity was inhibited in RAdMMP-13ASRz injected tumors as a result of inhibition of MMP-13 expression. In addition, the amount of proliferating Ki67 positive cells in the tumors injected with MMP-13 antisense ribozyme was reduced by 30%. These results show, that MMP-13 antisense ribozyme potently inhibits expression of MMP-13 by SCC cells and suppresses SCC tumor growth *in vivo*. In conclusion, these results show, that MMP-13 plays an important role in invasion, and survival of SCC cells *in vivo*.

050

Increased Levels of Phosphorylated Small Heat Shock Protein (Hsp27-p82) in the Epidermis of Patients with Keratin Genodermatoses

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Keratin gene mutations cause genetic disorders that produce phenotypes in various epithelia including the skin, hair and nail. Generally, mutations are located in the structurally sensitive α-helical core of the protein, which weakens the intermediate filament (IF) network. Subsequent stress placed on the epithelia then causes filament collapse and initiates a cellular ''stress'' response. It is the response of the damaged cells that characterises the resulting clinical phenotype. To further understand how these phenotypes develop and how similar mutations in the same gene can give rise to different phenotypes, we have studied several patients with different genodermatoses. We examined expression of inducible keratins (K6, K16 and K17) and two small heat-shock proteins (Hsp27 and αB-crystallin). In addition, we used an antibody specific for the phosphorylated ''active'' form of Hsp27 (Hsp27-p82). Skin biopsies from 5 normal volunteers, 3 patients with psoriasis (lesional and peri-lesional skin) and 8 patients with genodermatoses (1 EBS, 2 EHK, 1 EPPK, 2 IBS and 2 LEN) were studied. Keratin mutations were defined in all 8 patients. Fixed biopsies processed and sectioned at 5 µm, incubated with primary antibody (or control buffer) and the antigens visualised by immunohistochemistry with DAB. Sections were treated with primary monoclonal or polyclonal antibodies to K16, K17, Hsp27, Hsp27-p82 and αB-crystallin. In all eight genodermatosis cases examined, a large increase in the expression of K16, NF-IL6 and Hsp27 was observed at the site of cellular damage. In addition, levels of activated Hsp27-p82 were increased. There was more variable up-regulation of K17 and little effect on αB-crystallin. Thus, keratin mutations weaken the IF network and subsequent cellular stress collapses the filament network and invokes a stress response typified by increased expression of K16 and specific phosphorylation of the small heat-shock protein, Hsp27.

051

Differential Regulation of Apoptosis by Bcl-X Proteins in Human Melanoma Cell Lines

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Bcl-X, a member of the Bcl-2 gene family is expressed in two known splice variants, Bcl-X_S (proapoptotic) and Bcl-X_L (antiapoptotic) which regulate cell death in eukaryotic cells. Bcl-X proteins can form homo- and heterodimers with other Bcl-2-related proteins, their active form being localized in the outer mitochondrial membrane. Until now the mechanism of Bcl-X_S induced apoptosis is not clear. Expression analyses of Bcl-2-related proteins in melanoma cell lines by Western blotting revealed high levels of Bcl-X_L and Bcl-2 protein in melanoma cell lines, whereas Bcl-X_S was only weakly expressed. In order to investigate the role of Bcl-X_S and Bcl-X_L for apoptotic processes in human melanoma cell lines, we subcloned full-length cDNA fragments by RT-PCR. Identity of the clones was confirmed by sequence analysis. For overexpression, both cDNAs were further subcloned in sense and in antisense-orientation downstream of a tetracyclin-regulatable promoter and were transfected into established Tet-On (SK-Mel-13, Bro) and Tet-Off (Mel-2a) melanoma cell lines. Expression of Bcl-X_L/X_S after transient transfection was verified on the mRNA level by Northern and on the protein level by Western analysis, and apoptosis was quantified by the cell death detection ELISA (Roche). Induction of Bcl-X_S by doxycyclin led to significantly increased apoptosis as compared to noninduced cultures (1.2–1.7-fold), whereas induction of Bcl-X_L resulted in reduced apoptosis. Sensitivity to Fas ligand-induced apoptosis was strongly reduced in cell clones, stably transfected with Bcl-X_L. These findings suggest that exogenous Bcl-X_S may provide a novel tool for initiating cell death in human melanoma cells. Bcl-X_L is equipotent to Bcl-2 in prohibiting FasL-induced apoptosis in melanoma cells.

052 [Oral 007]

The Released Collagen XVII Ectodomain Inhibits Human Keratinocyte Migration

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Collagen XVII is a structural component of the hemidesmosomes and exists in two forms, as a 180-kDa type II transmembrane protein and as a soluble 120 kDa form, which corresponds to the extracellular collagenous domain of collagen XVII. We have previously shown that the release of the ectodomain from keratinocyte surface was generated by at least three members of the ADAM family. Until now, only little is known about the physiological and pathological relevance of the shed ectodomain. Time chase experiments with biotinylated keratinocytes revealed that the ectodomain was detectable within minutes and showed high stability in the medium for more than 48 h. The use of domain specific antibodies demonstrates that the authentic shedding product contains at least a part of the NC16A domain and the full C-terminus of the collagen XVII molecule. To get more information about the involvement of collagen XVII-shedding in the reepithelialization of healing wounds, we investigated the haptotactic migration of COS-7 cells on fibronectin. Collagen XVII transfected COS-7 cells showed a 2.5-fold increased motility compared to control cells, which were transfected with the empty vector. Transfection with a NC16a deletion construct of collagen XVII, which was not shed from the cell surface leads to a 35% increased migration rate compared to collagen XVII transfected cells. Addition of affinity purified collagen XVII-ectodomain (100 nM) to the lower chamber of the transwell plates leads to 40% reduced motility of both, full-length collagen XVII and NC16a deleted collagen XVII transfected cells. In addition, *In vitro* scratch assays with normal human keratinocytes were performed. Correspondingly, the addition of soluble collagen XVII ectodomain caused a significantly decreased rate of wound closure. The amino acid sequence of the ectodomain includes no putative integrin RGD-binding sites, but numerous, highly related KGD sequences, which have found in snake venom poisons and show similar integrin binding ability. These results suggests that the collagen XVII ectodomain includes integrin binding motifs for fibronectin, like the $\alpha_5\beta_1$ and α_V integrins. Recent studies revealed that these integrins were only expressed in migrating wound keratinocytes, but not in normal keratinocytes. Therefore, it is possible that the shed collagen XVII ectodomain plays a role in regulation of keratinocyte migration in wound healing processes.

053 [Oral 008]

Towards Gene Therapy of Recessive Dystrophic Epidermolysis Bullosa

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Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited skin disorder caused by mutations in the gene for collagen type VII. No conventional therapy is available for this disease, which justifies the perfection of treatments based on gene therapy. To this purpose, we have investigated the capacity of viral vectors to efficiently transduce a full-length collagen VII cDNA (> 9 kb) into human collagen VII-null RDEB keratinocytes *ex vivo*. Recombinant MoMuLV- and MSCV-based vectors efficiently transduced the keratinocyte cultures which led to the synthesis and secretion of collagen VII molecules of the expected size. Limited proteolysis of the recombinant collagen VII using pepsin or collagenase confirmed that the recombinant molecules are secreted as correctly folded helical trimers preserving their functional domains. Tri-dimensional skin equivalents (SE) were constructed using wild type or RDEB fibroblasts embedded substrates overlaid with wild-type, RDEB or reverted RDEB keratinocytes. After 15 days exposure of the organotypic cultures to the air–liquid interface, immunohistochemical analysis detected a strong linear staining of collagen VII along the BM in the SE formed by the RDEB fibroblasts and the reverted RDEB keratinocytes. After a 30-day exposure to air, the immunofluorescence signals was restricted to the BM and was indistinguishable from that obtained in the SE made with the wild-type cells. Our results show that (i) retroviral vectors efficiently express the large collagen VII cDNA, (ii) the recombinant collagen VII is efficiently synthesized, matured and secreted by the transduced cells; (ii) phenotypically reverted skin can be produced by the RDEB transduced keratinocytes. These findings pave the way to a preclinical validation of a gene therapy approach for the treatment of RDEB.

054 [Oral 047]

Matrix Metalloproteinase-19 Expression in Wound Healing and in Dermal Fibroblasts

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We have examined the expression of matrix metalloproteinase-19 (MMP-19) in cutaneous wound healing and the roles of mitogen-activated protein kinase (MAPK) signaling pathways in the regulation of MMP-19 expression in normal dermal fibroblasts. MMP-19 was expressed in human cutaneous wounds, in venous and decubitus chronic ulcers as well as in well granulating ulcers. MMP-19 protein was detected in wound stroma in endothelial cells and fibroblasts, often in the vicinity of inflammatory cells. Expression of MMP-19 in human skin fibroblasts was enhanced by IL-1, PDGF, TGF-β, and most potently by TNF-α. TNF-α activated extracellular signal-regulated kinase (ERK)1,2, Jun – N-terminal kinase (JNK) and p38 MAPK in dermal fibroblasts. Induction of MMP-19 mRNA expression by TNF-α was in part (by 50%) prevented either by blocking p38 activity by SB203580 or by blocking the activation of ERK1/2 by PD98059. Activation of endogenous ERK1/2 by adenovirus-mediated delivery of constitutively active MEK1 resulted in induction of MMP-19 expression. Activation of endogenous p38 alone by adenovirally delivered expression of constitutively active MKK3b and MKK6b also enhanced MMP-19 expression and augmented the up-regulatory effect of constitutively active MEK1 on the expression of MMP-19. Activation of JNK by adenovirus-mediated expression of constitutively active MKK7 also augmented the enhancement of MMP-19 expression by constitutively active MEK1, but had no effect alone. The abundant proMMP-19 production induced by simultaneous activation of ERK1/2 together with JNK or p38 was associated with proteolytic processing of proMMP-19. These results show, that MMP-19 is specifically expressed during cutaneous wound healing, and identify two distinct mechanisms for inducing MMP-19 expression in fibroblasts: AP-1-dependent activation via ERK1/2 pathway, and AP-1-independent activation via p38 pathway, both of which apparently control the proteolytic activity of normal fibroblasts, e.g. in wound repair and tumor invasion.

055 [Oral 057]

Proteinases of the BMP-1 Family Convert Procollagen VII to Mature Anchoring Fibril Collagen

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Collagen VII is located at the basement membrane zone of the skin. Here it is the major structural component of the anchoring fibrils which ensure the cohesion between the epidermis and the dermis. Collagen VII is secreted into the extracellular matrix as a precursor, procollagen VII. A C-terminal propeptide is cleaved during maturation of the anchoring fibrils. However, the mechanisms of anchoring fibril maturation, including the cleavage site and the enzyme processing procollagen VII have remained elusive. Genetic evidence has suggested the involvement of BMP-1 in this process, since a naturally occurring deletion in the *COL7A1* gene, 8523del14, which eliminates a putative cleavage site for BMP-1, prevents processing of procollagen VII in the skin. Here we show that recombinant BMP-1 cleaves full-length human procollagen VII *in vitro,* yielding a cleavage product of the same size as mature collagen VII in the dermis. In order to determine the cleavage site, a truncated recombinant procollagen VII containing an intact C-terminus was produced. This construct was cleaved by both BMP-1 and the related proteinases mammalian tolloid-like-1 and –2 (mTLL-1 and mTLL-2) at the predicted site *In vitro*. Analysis of collagen VII in the skin of BMP-1 deficient mouse embryos demonstrated that procollagen VII was processed to the same extent compared to wild type embryos. This suggests that *in situ* the collagen VII precursor can be cleaved by at least three metalloproteases including BMP-1, mTLL-1 and mTLL-2.

056

Withdrawn

057

Dystroglycan, a Transmembrane Component in Cutaneous Cells, is Proteolytically Processed

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Dystroglycan (DG) is a membrane spanning complex composed of two subunits, α and β dystroglycan. Originally, DG was identified as an integral component of the dystrophin glycoprotein complex (DGC) which provides a tight link between the cytoskeleton and the basement membrane (BM) in muscle. Dysfunction of the DGC has commonly been implicated in the pathogenesis of severe forms of neuromuscular diseases. More recent work showed that DG is also expressed in a variety of nonmuscle tissues like skin, peripheral nerve, kidney and lung. Despite of expanding knowledge about the function of DG in nonmuscle tissues a number of questions remained to be answered. Therefore we analyzed the biological function of DG in skin and cutaneous cells in more detail. In order to study the expression and localization of DG in skin and cutaneous cells on the protein level we generated domain-specific antibodies for α and β DG and used them for immunofluorescence studies and Western blotting (WB). In parallel DG was analyzed on the mRNA level by *in situ* hybridization. DG is present at the skin BM and is synthesized by keratinocytes and fibroblasts, *In vitro*. WB of cell extracts and media of keratinocytes revealed that the transmembrane protein β DG is localized in the cell extracts whereas α DG was only detected in the media. In recent studies the existence of a truncated form of β DG was described and it was suggested that this truncated 30 kDa fragment of β DG is generated by proteolytic processing of the extracellular domain of β DG. We showed that in keratinocytes this processing of β DG takes place and can be stimulated by PMA, EGF and Il-1 β. Furhtermore, this process is sensitive to hydroxamate inhibitors which indicates that the cleavage is mediated by matrix metalloproteinases or ADAM's. In order to analyze the effect of ligand interaction on the shedding process we examined perlecan −/− fibroblasts. In these cells, which lack a strong binding partner for α DG, the processing of β DG was strongly enhanced. This study shows that DG is constitutively shed from the cell surface in keratinocytes and fibroblasts. The shedding has a great impact on the integrity of the epithelial DG complex.

058

A Role of Dermatopontin in Wound Healing

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Dermatopontin (DPT) knockout (KO) mice, generated by targeted disruption of *Dpt* gene, display abnormal collagen fibrillogenesis and increased elasticity in skin. We have also shown the lack of DPT may cause decreased collagen accumulation in skin. We think DPT-KO mice could represent a useful animal model to investigate the wound healing, fibrosis, and tumorogenesis. DPT and fibril-associated genes expression levels and distributions during skin wound healing were assessed by RT-PCR and *in situ* hybridization. Samples were collected from wild-type mice wounds at 1 day, 4 day, 1 week, 2 week, and 3 week after full-thickness skin injury with 6 mm punches. Whereas very little expression was noted in unwounded skin, *Dpt* expression was up-regulated at 1 week after injury. Expressing cells were localized in fibroblastic cells throughout the granulation tissue. The pattern of *Dpt* expression was correlated with that of fibril-associated genes and type 1 collagen expression. Healing wounds were comparable between wild-type and DPT-KO mice during the repair process when analyzed using light microscopy. However, electron microscopic studies displayed alterations in collagen fibril formation of dermal wound in DPT-KO mice. These results suggest that DPT may function in remodeling wound matrix in a process that involves fibrillar collagen organization.

059

Modulation of Collagen Synthesis and Messenger RNA by Continuous and Intermittent Use of Topical Glucocorticoid

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Glucocorticoids have been shown to down-regulate collagen synthesis in human skin *in vivo*, thereby contributing to skin atrophy. The aim of the present study was to compare the effects of continuous and intermittent use of topical hydrocortisone on skin collagen synthesis and, furthermore, to elucidate the mechanism of collagen synthesis reduction induced by hydrocortisone. Collagen propeptides reflecting the synthesis rate of type I and III collagens were studied from suction blister fluids in nine healthy subjects after three weeks of continuous (twice daily) or intermittent (on three consecutive days weekly) topical hydrocortisone treatment and two weeks after the termination of treatment. Type I collagen mRNA was studied in the same subjects from skin biopsies by using *in situ* hybridization after three weeks' treatment. Three weeks' continuous treatment decreased the types I and III collagen propeptides in suction blister fluid by 89% and 82%, while intermittent treatment resulted in a corresponding decrease of 53% and 50%. *In situ* hybridization (ISH) studies from skin biopsies showed type I collagen mRNA to be markedly reduced in fibroblasts after continuous and intermittent steroid treatment. After a two-week drug-free interval, the synthesis rate was completely restored in both areas, and some subjects even showed up-regulation of synthesis in previously steroid-treated skin. As a conclusion continuous hydrocortisone for 3 weeks decreases markedly collagen propeptides and corresponding mRNA in human skin. Intermittent hydrocortisone has a less marked effect on the collagen synthesis rate.

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Minocycline Does Not Alter Collagen Type I Metabolism of Dermal Fibroblasts in Culture

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Antibiotic drugs such as Penicillamine, Penicillin G and Minocycline are used in the treatment of Systemic Sclerosis (SSc). However, there is little knowledge on the mechanism of antifibrotic action of these drugs. In general, excluding cell proliferation data, the influence of antibiotics on the metabolism of eukaryotic cells is poorly investigated. Penicillin G that may improve skin status of SSc-patients significantly did not influence collagen metabolism *In vitro* during previous investigations. Minocycline is another drug that may improve the clinical phenotype of SSc. We studied the effects of Minocycline by analysing the influence of various concentrations of Minocycline on cell proliferation, synthesis and degradation of collagen by human dermal fibroblast from healthy donors and SSc-patients (collagen high producer). More detailed, collagen metabolism of cultured dermal fibroblasts was studied by Northern Hybridisation for mRNA of collagen I, proline-4-hydroxylase, lysyl-hydroxylase, matrix metalloproteinase I and determination of collagen content in culture supernatants. Minocycline did not alter the expression of the investigated mRNA, independently on the dosage and the incubation times used. The amount of collagen I protein was not influenced. In conclusion, there is no evidence of a direct antifibrotic effect of Minocycline on dermal fibroblasts. Therefore, other mechanisms might be responsible for its effect in the treatment of SSc-patients. In the case of Minocycline, the anti-inflammatory action might be relevant for the positive effects known from SSc therapy.

061

Vasoactive Intestinal Peptide Stimulates Nitric Oxide Production by Mastocytosis Fibroblasts

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Nitric oxide (NO) and stress have been implicated in the pathogenesis of manyinflammatory and hyperproliferative disorders. It is also suggested that fibroblast–mast cell interactions, via their numerous mediators, play some role in cutaneous mastocytosis course. So, the aim of our study was to evaluate NO production by mono- and cocultures of fibroblasts and mast cells upon vasoactive intestinal peptide (VIP) stimulation. The following cells were used: skin fibroblasts from the lesions of urticaria pigmantosa patients with confirmed diagnosis of systemic mastocytosis, normal skin fibroblasts (controls), human leukaemic mast cell line (HMC-1) as either mono-cultures or cocultures of mast cells and fibroblasts (third passage). Cells were stimulated with different concentrations of VIP (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M). NO production was assessed by Griess method. Measurements were performed in quadruplicate. We observed that VIP stimulated NO production by mastosytosis fibroblasts and exert rather inhibitory effect on control ones in a dose-dependent manner. With increasing VIP concentrations – NO production was as follows: 0.97 vs. 1.46 μM, 1.36 vs. 1.82 μM, 1.46 vs. 1.94 μM, 1.46 vs. 1.99 μM, respectively. NO synthesis by HMC-1 cells was also inhibited by VIP and ranged from 1.44 to 1.64 μM and did not reach spontaneous production levels (mean 1.79 μM). As for cocultures, in control experiments NO production oscillated around levels observed on spontaneous NO production (no VIP stimulation) – mean 1.77 vs. 1.84 μM, respectively. However, in cocultures of mastocytosis fibroblasts and HMC-1 line NO production was significantly higher when comparing to control experiments and ranged from 2.04 to 4.02 μM (mean 3.33 μM). It seems that VIP is involved in modulatory processes of NO production by mastocytosis skin fibroblasts.

062

Inhibitory Effect of Substance P and Vasoactive Intestinal Peptide on Psoriasis Vulgaris Fibroblasts

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It is known that neuropeptides are involved in pathomechanism of psoriasis vulgaris. It is also suggested that mast cell–fibroblast interactions play a role in psoriatic lesions development. So, the aim of our study was to examine influence of substance p (SP) and vasoactive intestinal peptide (VIP) on the proliferation of fibroblasts obtained from the psoriatic lesions (PsLF), their close surroundings (PsSF), normal healthy skin of the control subjects and human leukaemic mast cell line (HMC-1). After 72-h stimulation of already selected concentrations of neuropeptides (VIP – 10^{-8}, 10^{-6} M and SP 10^{-13}, 10^{-8} M), proliferation assays were performed. In cocultures HMC-1 were preincubated with mitomycin C. Experiments were performed in quadruplicate. Fibroblasts from the fourth passage were used. We observed that spontaneous proliferation of PsLF was considerably higher than proliferation of PsSF and control ones – mean 2907 DPM vs. 2371 and 964 DPM, respectively. We also noticed that both VIP and SP inhibited proliferation of all the studied fibroblasts in an inversely dose-dependant manner. As for fibroblast-mast cell cocultures, inhibitory effect of neuropeptides was preserved when HMC-1 line cells were pretreated with mitomycin C. In cocultures, both neuropeptides exhibited slightly less inhibitory effect on cell proliferation. However, diseased fibroblasts (both PsLF and PsSF ones) were more susceptible to VIP and SP inhibition than control ones. HMC-1 presence seems to diminish the inhibitory effect of neuropeptides on control fibroblasts. However, as regards PsLF and PsSF ones, HMC-1 cells are unable to abolish this inhibitory effect. These observations suggest that in psoriasis vulgaris interactions between mast cells and fibroblasts seem to be somewhat disturbed, maybe by the previous (*in vivo*) nerve-mast cell influence.

063

Role of Boron and Manganese in Wound Healing Through Induction of MMP-2 and MMP-9

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Synthesis of new tissue and maturation of the wound by the remodelling of extracellular matrix (ECM) are two phases of wound healing which involved matrix metalloproteinases (MMPs). Clinical studies aim to demonstrate the beneficial effect of trace-elements in cutaneous wound healing. Thus, boron compounds would enhance wound healing by their action on extracellular matrix. In the same way, previous studies lead in our laboratory demonstrated an induction of integrin expression by keratinocytes after a treatment with manganese. Leif R Lund *et al* have demonstrated the presence as well as the localisation of MMP-2 and MMP-9 mRNA which play an important role during cutaneous wound healing. In our study, we observed the effect of boron and manganese on MMP-2 and MMP-9 keratinocyte expression. Human normal keratinocytes were cultured in monolayers and incubated during 24 h with boron (Labcatal, France) (0,5–1 – 5–6,46–10 μg per ml) or manganese (Labcatal) (0,1–0,2–0,3–0,6–1,5 μg per ml). MMP-2 and MMP-9 extracellular expression was studied using zymography on culture supernatants and Western blot on cell lysates and culture supernatants. RT-PCR was used for the study of mRNAs. After 24 h of incubation a significant increase of MMP-2 and MMP-9 secretion by keratinocytes incubated either with boron or manganese was obtained. In a similar manner, an increase of intracellular expression of pro-MMP9 using Western blot was noted associated with an increase of MMP-9 mRNA expression in keratinocytes cultured with boron or manganese. In regard of MMP-2 mRNA localisation in dermis underlying the wound and knowing that MMP-9 is involved in keratinocyte migration from the wound edges (Leif R Lund *et al*), our results demonstrate that one mechanism of wound healing of boron and manganese observed *in vivo* is the induction of MMP-2 and MMP-9 production *in situ*.

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Age-Related Decrease of L-Ascorbate Uptake by Human Skin Fibroblasts: Regulation by a Bertholletia Excelsa Pericarp Extract

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Cellular L-ascorbate (LA) homeostasis plays a determinant role in neutralizing free radicals formed during oxidative metabolism or UV exposure of human skin, radicals suspected to trigger oxidative stress-related disorders and ageing at the molecular, cellular and tissue levels. LA is also the main cofactor for the prolyl- and lysyl-hydroxlases involved in co- and post-translational collagen modifications and stimulates collagen biosynthesis at the protein and mRNA levels. It has been previously shown that fibroblasts from a sun exposed area, where wrinkles appear easily, present a decreased of collagen type I and III synthesis and a lower response to LA stimulation. Primary cultures of normal human skin fibroblasts were obtained from face lifts (12 healthy caucasian women from 37 to 74 years old) and grown in an E-199 medium containing 2 mM L–glutamine and 10% v/v foetal calf serum. LA uptake was carried out using a 2-h incubation with 150 μM ^{14}C-LA (specific activity 14.1 Ci per μM) then quantified by liquid scintillation after separation by NH_2 phase HPLC. Cellular proteins were measured using the bicinchoninic acid reagent. An age-related decrease of LA uptake was observed on fibroblast cultures (from 4 to 1.5 pmol LA per μg protein). Furthermore, a Bertholletia excelsa pericarp extract, enriched in ellagic acid and O-methylellagic acid, was found to increase the fibroblast input of LA (+ 55% at 1 μg per ml to + 78% at 5 μg per ml) and thus appears to be of potential interest in aging treatment.

065

Retinoid Regulation of Collagen Synthesis by TGF-β1, CTGF, and Cyr61 in Chronologically Aged and Photoaged Human Skin *In Vivo*

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Synthesis of type I procollagen (COL), the major structural protein in skin, is reduced in aged and photoaged human skin. TGF-β is a major regulator of COL biosynthesis. Recently, two related proteins, CTGF and Cyr61, have been demonstrated to modulate TGF-β regulation of COL synthesis. CTGF is directly regulated by TGF-β, and like TGF-β promotes COL synthesis. Cry61 acts in opposition to TGF-β and CTGF to reduce COL synthesis. We have examined TGF-β, CTGF, and Cyr61 expression, and their regulation by retinoid, in aged and photoaged human skin. Quantitative real-time RT-PCR analysis demonstrated that TGF-β1 (but not TGF-β2 or β3) and CTGF mRNA levels, normalized to total RNA or housekeeping gene 36B4, were reduced in sun-protected aged (80+ years) skin by 65% and 45%, respectively, compared to young skin (20–30 years, $n = 6$, $p < 0.05$). In photoaged forearm skin, TGF-β1 and CTGF mRNA were reduced by 60% and 50%, respectively, compared to subject-matched sun-protected skin ($n = 6$, $p < 0.05$). In contrast, Cyr61 mRNA levels were increased 3.9-fold in aged skin ($n = 5$, $p < 0.05$), and 2.9-fold in photoaged skin ($n = 3$, $p < 0.05$). Immunohistology revealed that TGF-β1 and CTGF proteins were expressed throughout the epidermis and dermis, while Cyr61 was expressed mainly in dermal cells. Topical application of retinol (1%) for seven days markedly increased TGF-β1 (2.3-fold), CTGF (2.6-fold), and COL (2.6-fold) gene expression, compared to vehicle ($n = 5$, $p < 0.05$) in aged skin. In contrast, retinol treatment reduced Cyr61 gene expression by 65% ($n = 6$, $p < 0.05$). Similarly, retinol increased TGF-β1 (2.1-fold), CTGF (2.2-fold), and COL (3.0-fold) ($n = 4$–6, $p < 0.05$), and reduced Cyr61 gene expression by 70% ($n = 3$, $p < 0.05$) in photoaged skin. *In situ* hybridization revealed increased TGF-β1 and CTGF mRNA in the dermis and epidermis of aged and photoaged skin. These data reveal that molecular mechanisms that mediate reduced COL synthesis are strikingly similar in aged and photoaged skin. These molecular mechanisms involve aberrant expression of both positive (TGF-β, CTGF) and negative (Cyr61) regulators, whose expression is normalized by topical retinoid treatment.

066

Chemokines: Key Regulators in the Dynamic Process of Cutaneous Wound Healing

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Wound healing represents a dynamic process involving directional migration of leukocytes and structural cells such as keratinocytes, fibroblasts, and endothelial cells. Recently, it has been shown that chemokines regulate leukocyte trafficking. We performed a comprehensive analysis of cytokine, adhesion molecule, matrix metalloproteinase (MMPs), growth factor and chemokine expression in murine cutaneous wound healing using real time quantitative PCR ($n = 162$ different genes). Compared to other cytokines, growth factors and MMPs, chemokines were the most highly regulated genes. Chemokine expression coincided with the appearence of matching receptors. Within hours after injury, chemokines such as MIP-2, LIX, MIP-1α, MIP-1β, MIP-1γ, eotaxin, MCP-1, MCP-3, and IP-10 showed peak levels of expression. However, MCP-6, SDF-1 and RANTES dominated chemokine expression on day 2–5; while MIG and CCR6 were up-regulated on day 5–10. Next to leukocytes, we could show that resting as well as activated human primary keratinocytes (CXCR1, CXCR2, CCR6), dermal fibroblasts (CCR7) and dermal microvascular endothelial cells (CXCR3, CCR3) express a distinct and functionally active repertoire of chemokine receptors. Taken together, our results indicate that chemokines may regulate the directional migration of both leukocytes and structural cells in the dynamic process of wound healing. These findings may have impact on the development of novel therapeutic strategies for the treatment of chronic ulcers.

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Increase of Melanoma Cells Invasion by Elastin Derived Peptides Through Up-Regulation of MMP-2 Activation

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Elastin, as a main constituent of elastin fibers, plays an important role in skin biology. Its degradation by gelatinases (MMP-2, MMP-9) generates elastin peptides (E.P.) which exhibit several biological functions. Particularly, interaction between E.P. and a truncated β galactosidase elastin/laminin receptor present at the plasma membrane of various cell types was shown to trigger MMP-expression. Here, we studied the effects of a soluble elastin-derived peptides (EDPs) from alkaline or elastase hydrolyses of insoluble elastin, on melanoma cells invasion and MMP expression. When melanoma cells were cultured on EDPs coated dishes, the expression of MMP-2, MT1-MMP, TIMP-2, but not MMP-1, were enhanced. The stimulatory effect of MMP-2 production could be reproduced when melanoma cells were grown on dishes coated with Val-Gly-Val-Ala-Pro-Gly (VGVAPG) peptide, an elastin derived hydrophobic hexapeptide which represented the elastin receptor binding sequence of tropoelastin. Furthermore, EDPs did not altered nor melanoma cells adhesion nor their proliferation, suggesting that the observed phenomenon required transcriptional or post-transcriptional mechanisms. In a previous work, we have shown that the activation of MMP-1 and MMP-2 occurred within a three dimensional type I collagen matrix, thus allowing melanoma cells invasiveness. In presence of EDPs, MMP-2 activation was strikingly exacerbated, related to a decrease of TIMP-2 production. In the same manner, invasion of type I collagen matrix by melanoma cells was significantly increased. These results, in keeping with our previous data suggest that the main fibrillar matrix macromolecules of human dermis i.e collagen and elastin, actively contribute to melanoma cells invasion, through modulation of MMP-2 activation.

068

5-Fluorouracil Blocks TGF-β-Induced α((2)) Type I Collagen (COL1A2) Gene Expression in Human Fibroblasts: Role of JNK/AP-1

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5-fluorouracil (5-FU), a pyrimidine analog, is widely used in cancer chemotherapy and for its antifibrotic effect in the glaucoma surgery. Recently its interest in the treatment of keloids, scars which overgrow the boundaries of original wounds, has been suggested. Given the physiopathological importance of TGF-β in keloid formation, we focussed our attention on the molecular events underlying the effect of this drug on TGF-β-induced type I collagen gene expression. Western blot analysis and transient transfections experiments demonstrated that short-term 5-FU applications (10 μM, 24 h) antagonize TGF-β driven COL1A2 transcription, in human dermal and lung fibroblasts. EMSA experiments showed that 5-FU inhibit the formation of Smad/DNA complexes upon TGF-β stimulation. Given the critical role of of c-Jun in mediating the antagonistic effect of TNF-α on the up-regulation of type I collagen gene expression by TGF-β, we investigated the effect of 5-FU on cJun/AP-1 signaling pathway. Transient transfection experiments demonstrated that 5-FU (10 μM, 24 h) is able to transactivate the AP-1 specific promoter construct pAP1-lux. In EMSA, a strong band identified as an AP-1-containing complex was detected with nuclear extracts from 5-FU-treated fibroblasts, which was supershifted with a cJun antibody. On the other hand, we demonstrate that in a cellular context devoid of JNK activity (i.e. *JNK*$^{-/-}$ fibroblasts), 5-FU do not inhibit the formation of Smad/DNA complexes and resulting Smad-driven COL1A2 transcription in response to TGF-β. Together, these results suggest a critical role for JNK-mediated c-Jun phosphorylation in mediating the inhibitory effect of 5-FU on TGF-β/Smad signaling and subsequent up-regulation of COL1A2 gene expression by TGF-β. In addition to the effect of 5-FU long-term exposures on cellular metabolism, the antagonistic activity of 5-FU against TGF-β/Smad-driven COL1A2 gene transcription could explain the clinical effect of 5-FU in keloids.

069 [Oral 058]

An Amino Acid Substitution in Integrin α6 Causes Junctional Epidermolysis Bullosa with Pyloric Atresia

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Mutations in the genes for integrin α6β4 cause junctional epidermolysis bullosa associated with pyloric atresia (PA-JEB), an inherited disorder characterized by severe blistering of the skin, pyloric occlusion and aplasia cutis. In this study, we report the characterization of a consanguineous PA-JEB family with a member affected by a lethal form of the condition. The proband's skin was not reactive with the antibodies specific to integrin α6β4. Western blot and immunoprecipitation analysis of cellular extracts obtained from cultures of the patient's keratinocytes revealed expression of integrin β4 and failed to detect the mature form of integrin α6. The steady-state level of the α6 and β4 transcripts in the PA-JEB keratinocytes resulted comparable to that of wild type controls. Genetic analysis of the α6 cDNA revealed a homozygous base pair substitution at position 286 that results in the substitution of a serine to a leucine residue (286 S-to-L). Analysis at genomic level assessed the mendelian transmission of the DNA sequence variation in the PA-JEB kindred. The 286 S-to-L substitution is localized in an amino acid sequence that is conserved in most of the integrin α subunits and is involved in the folding of the N-terminal region of the α6 integrin into a β-propeller domain. Transfer of the mutant α6 cDNA into α6-null keratinocytes confirmed the deleterious effect of mutation 286 S-to-L on the expression of the abnormal α6 subunit. Biochemical analysis of the patient's keratinocytes demonstrated that substitution 286 S-to-L is sufficient to trigger the massive proteolytic degradation of the α6 integrin and showed that the lysosomal pathway is implicated in the proteolytic process. Our results show for the first time that the conformation alteration of extracellular domain of α6 integrin induced by a single amino acid substitution triggers the massive degradation of integrin α6β4 and give rise to a lethal PA-JEB phenotype.

070

Deletion of a Cytoplasmic Domain of Integrin β4 Causes Epidermolysis Bullosa Simplex

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Integrin α6β4 is a hemidesmosomal transmembrane molecule involved in maintaining basal cell–matrix adhesion through interaction of the large intracytoplasmic tail of the β4 subunit with the keratin intermediate filament network, at least in part through its binding with plectin and BP180/type XVII collagen. Here we report a patient with predominant features of epidermolysis bullosa simplex due to a mutation in the integrin β4 gene. The patient, a 49-year-old-female, had mild acral blistering from birth on, dystrophy of the nails with onychogryposis, and enamel hypoplasia. She had no alopecia and no history of pyloric atresia. Electron microscopy and antigen mapping of a skin blister revealed that the level of separation was intraepidermal, low in the basal keratinocytes between the inner and outer attachment plaque of the hemidesmome. Immunofluorescence microscopy revealed absent binding of monoclonal antibody 450–11 A against the third fibronectin III repeat on the intracellular domain of integrin β4, whereas binding was reduced with monoclonal antibodies recognizing epitopes on the extracellular domain. At the molecular level the phenotype was caused by a novel two base-pair deletion 4733delCT in *ITGB4*, resulting in-frame skipping of exon 36 and a deduced 50 amino acid deletion (1573–1622) within the third fibronectin type III repeat in the cytoplasmic domain of the integrin β4 polypeptide. Immunoblot analysis demonstrated a 5-kDa shorter β4 polypeptide. The 4733delCT mutation was heterozygously present in the DNA. The patient is also expected to be heterozygous for a null allele, since no full size protein was detected *In vitro* and the epitope 450–11 A was absent *in vivo*. This case learns us that deletion of the third fibronectin type III repeat in the cytoplasmic domain of integrin β4, which is thought to interact with BP180/type XVII collagen, is clinically pathogenic and results in a mild phenotype with predominant features of epidermolysis bullosa simplex.

071

Experimental Mouse Model of Bullous Pemphigoid Using Antibodies Generated Against an Antigenic Epitope of Hemidesmosomal Plaque Protein BP230

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Bullous pemphigoid (BP) is an IgG-mediated autoimmune blistering disease targeting the hemidesmosomal plaque protein BP230 and the transmembrane protein BP180. Immunodominant and pathogenic epitopes associated with BP have been mapped to the extracellular domain of BP180, but the pathogenetic role of anti-BP230 antibodies remains unclear. To investigate this phenomenon, rabbit polyclonal antibodies were generated against the antigenic segment of the human BP230 antigen (BPAG 1, 1914–34), a sequence which shows 74% homology in human and mouse BP230 protein. The rabbit polyclonal immune serum was tested on human and mouse skin by indirect immunofluorescence and immunoblotting, and also by the ELISA technique, using antigenic epitope-coated wells. The specificity and titres of the rabbit immune serum (1:2840) were suitable for passive transfer experiments. The purified rabbit IgG from immunized rabbit serum was transferred subcutaneously into the dorsal skin of neonatal CBA mice (n = 7) in a dose of 5 mg IgG per 50 μL. After 6 h, the dorsal skin areas of the injected animals were markedly erythematous, whereas in the control animals injected with PBS or control rabbit IgG, the sites of injection cleared up. After 24 h, one animal exhibited a small blister clinically, but the dorsal skin areas of all the injected animals were constantly erythematous and upon gentle friction produced fine persistent wrinkling of the epidermis. Mice were then sacrificed and immunohistological examinations revealed linear rabbit IgG deposition along the basement membrane zone of the perilesional mouse skin, and in 5 animals subepidermal blister formation was also demonstrated. The rabbit IgG deposition labelled the epidermal roofs of the vesicles. Haematoxylin-eosin stained sections showed the separation of the epidermis from the dermis, producing a subepidermal vesicle. A slight intradermal inflammatory reaction was also detected. These findings demonstrate that antibodies against an antigenic epitope of BP230 protein can cause subepidermal blister formation in neonatal mice.

072

Paraneoplastic Pemphigus Sera Display Plakophilin-3 Autoreactivity

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Paraneoplastic pemphigus (PNP) is a not well-understood auto-immune mucocutaneous bullous disorder appearing in association with neoplasia. Classically, patients with PNP develop autoreactivity against desmosomal plaque proteins of the plakin family like desmoplakin1/2, envo- and periplakin and plectin, against desmosomal cadherins like desmoglein 1/3 and also against a yet unidentified 170 kDa protein. In this study we tested whether PNP is associated with autoreactivity against another intracellular desmosomal protein namely the armadillo-repeat containing plakophilin-3 (PKP3) protein. HEK293 cells were transiently transfected with a pEF6/myc-his and a pEGFPN2 construct encoding human PKP3 (85 and 115 kDa *Mr*, respectively) and protein lysates were made in Laemmli buffer. Lysates underwent 7,5% SDS-PAGE and Western blots were subsequently labelled with 5 PNP sera, 4 PV sera, 2 PF sera, 5 BP sera, 1 CP serum and 1 LAD serum. A mouse monoclonal anti-PKP3 antibody raised against a 20 AA peptide of human PKP3 was used as a positive control. Autoreactivity against, respectively, 85 and 115 kDa recombinant PKP3 protein products was detected in all 5 PNP sera and in 1 PV serum. None of the basement membrane zone bullous diseases reacted with these proteins. The presence of autoantibodies against PKP3 in PNP-sera was subsequently confirmed on human epidermal lysate blots. This is the first report of PKP3 reactivity in bullous disorders, seemingly consistently present in the patients with PNP tested. The presence of PKP3 reactivity in one PV patient is not surprising, since also in this disease the desmosome is targeted. We are currently investigating whether PKP-3 autoreactivity is merely a secondary phenomenon induced by keratinocyte injury or whether it can act primarily to induce blisters.

073

Animal Models for Skin Blistering Conditions: Absence of Laminin 5 Causes Hereditary Junctional Mechanobullous Disease in the Belgian Horse

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Recent achievements in the genetic correction of keratinocytes isolated from patients with junctional epidermolysis bullosa (JEB) have brought somatic gene therapies within reach. Because gene therapy protocols require preclinical validation in animals, we have elucidated the genetic basis of the hereditary junctional mechanobullous disease in the Belgian horse, a condition characterized by blistering of the skin and mouth epithelia, and loss of the hoof. Immunofluorescence analysis associated the condition to the absent expression of the $\gamma 2$ chain of laminin 5 and designated *lamc2* as the candidate gene. Comparative analysis of the nucleotide sequence of the full-length $\gamma 2$ cDNA isolated by RT-PCR amplification of total RNA purified from the epithelium of a JEB foal and a healthy control disclosed the homozygous basepair insertion 1368insC in the affected animal. Mutation 1368insC results in a downstream premature termination codon and is predicted to cause absent expression of the laminin $\gamma 2$ polypeptide. Our results also show that: (1) the horse JEB genetically corresponds to the severe Herlitz form of JEB in man; (2) the amino acid sequence and structure of the horse laminin $\gamma 2$ chain are virtually identical to the human counterpart; (3) the moderate eruption of skin blisters in the affected animals with respect to the human H-JEB patients correlates with the protection provided by hair. Our observations demonstrate that the affected foals are a convenient source of epithelial cells from tissues that cannot be obtained from human JEB patients, and suggest that hairless strains of animals with recessive skin disorders would be the best models for *in vivo* gene therapy approaches of skin blistering diseases.

074

Dose-Effect Relation in SJS or TEN Related to Allopurinol

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By opposition to idiosyncratic reactions, allergic reactions are generally considered dose-independent. In this debate, we have been interested by studying the relation between the drug dose and the risk of SJS (Stevens–Johnson syndrome) and TEN (toxic epidermal necrolysis). We used the database of EuroSCAR, an international ongoing case-control surveillance of severe cutaneous adverse reactions. The data of this presentation concern patients included between 07/1997 and 07/2001 (370 cases of SJS/TEN and 1308 controls). Many causative drugs were identified. For our study of the dose-effect relation, we selected a drug frequently used at different doses and to which a sufficient number of cases included in EuroSCAR had been exposed. Allopurinol met both criteria. We identified 75 cases and 31 controls exposed to allopurinol (odds ratio 10.7, 5.9–19.6) Doses missed or were unprecise for 33 cases and 16 controls. Weight missed for another case patient. Median doses were 300 mg per day for cases and 100 mg for controls. Mean doses were significantly higher in cases (235 mg per day) than in controls (143) ($p < 0001$) Range was nearly the same. Odds ratios were 1.8 (0.6–5.2) for ≤ 100 mg per day, and for doses > 100 mg per day and 28.1 (10.9–72.1) for higher doses. Patient weight was not a bias, the mean doses by kg were higher in cases than in controls (3.53 mg per kg per day vs. 1.91, $p < 0.001$). Prior data already suggested that high doses of medication could be related to higher risk of ADR's. For example, in AIDS patients, the rate of reactions to cotrimoxazole were higher for high doses used for treating pneumocystosis than for low doses used in prevention. In conclusion, we consider that the dose is a major risk factor for allopurinol-induced SJS or TEN. Further studies are needed to verify this finding with other drugs.

075

Detection of Circulating IgG and IgA Antibodies against Pulmonary Tissue in Paraneoplastic Pemphigus

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Progressive respiratory failure with bronciolitis obliterans might lead to death in patients with paraneoplastic pemphigus (PNP) especially in cases with lichenoid skin symptoms. Possible causes of the respiratory failure include infection, toxic effects induced by chemotherapy, neoplasia and autoantibody-mediated pulmonary injury. Previously G. Anhalts group detected IgG in the pulmonary tissue of severe PNP cases, however, the binding site of circulating IgG has not been determined and its antigenicity remained obscure. Blood samples of two immunologically verified PNP patients with severe pulmonary involvement have been studied. Both patients had lichenoid skin symptoms, extended erosive stomatitis, laryngitis and corneal erosions as well as severe dyspnoe. Although both skin and mucosal symptoms got into remission under high doses of immunosuppressive treatment and/or surgical excision of the underlying tumor, the dyspnoe showed in both cases rapid progression leading to death. Both circulating IgA and IgG bound to rat pulmonary tissue along the bronchial respiratory epithelial cell surfaces. Control sera didn't show similar reactivity. The detection of circulating autoantibodies against respiratory epithelium suggests, that these antibodies might have a pathogenic role in the pathomechanism of pulmonary injury in PNP.

076 [Oral 006]

Osteopontin Polarizes Dendritic Cell Maturation Towards a Dendritic Cell 1 Phenotype

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Immature dendritic cells (DC) are located at epithelial borders. After activation and antigen uptake they mature and migrate into secondary lymphatic organs to initiate T-cell mediated immunity. As DC1 (high secretion of IL-12) or DC2 they are able to polarize naive T-cells towards either a Th1 or Th2 phenotype, decisively affecting the outcome of an immune response. Recently we demonstrated that Osteopontin (OPN) is important in cutaneous contact hypersensitivity by guiding DC into lymph nodes. Since OPN has been identified to have Th1 cytokine like properties, we now explored the effect of OPN on the phenotypic and functional maturation of DC. Human monocyte derived DC were cultured +/− OPN and supernatants and cells were analyzed after 24 and 48 h by ELISA or FACS. OPN strongly induced DC activation, up-regulating their expression of HLA-DR, CD40, CD80, CD86, CD44 and CD56 and their TNF-α secretion. In allogeneic mixed lymphocyte reactions (MLR) OPN was either added directly into MLR with immature DC or OPN prestimulated DC were used. When OPN was added to MLR with immature DC, T-cell proliferation was enhanced, while addition of OPN to T-cells alone had no effect on their proliferation or cytokine secretion. However, when MLR was performed with OPN activated DC a strongly increased T-cell proliferation was detected compared to control DC. In MLR supernatants we found that both addition of OPN to MLR or OPN prestimulation of DC induced an up-regulated secretion of the Th1 cytokine IFN-γ. Furthermore, high amounts of IL-12p70 were detected when OPN was added to the MLR, while IL-10 was not modulated. Our findings indicate that OPN induces DC maturation and their polarization towards a DC1 phenotype, indicating that the Th1 cytokine like properties of OPN are at least in part mediated through its effect on DC.

077 [Oral 021]

Dendritic Cell-Derived Interleukin 13, Induced by Exposure to Protein-Allergens and Subsequent Activation of STAT6, is a Key Factor for the Acquisition of their Capability to Induce Th2 Cytokine Production

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Dendritic cells (DC) are able to induce not only Th1 but also Th2 immune responses after stimulation with allergens. While DC-derived IL-12 and IL-18 are the key factors for the induction of Th1 cells, early signals being involved in Th2 differentiation are less well characterized so far. To analyze such early signals we used an antigen-specific setting with $CD4^+$ T cells from atopic donors stimulated in the presence of autologous mature DC, which were pulsed with different allergen doses. The addition of increasing amounts of allergen during DC maturation with TNF-α, IL-1β and prostaglandin E_2 resulted in enhanced secretion of IL-6 and IL-12 by DC followed by increased production of Th1 (IFN-γ) as well as Th2 (IL-4, IL-5) cytokines by $CD4^+$ T cells. The coculture of allergen-treated DC and $CD4^+$ T cells also led to a dose-dependent expression of active STAT6 which was visible already after 1 h. Additionally, rapid phosphorylation of STAT6 was seen in immature DC after stimulation with allergens but not with LPS or HSA. STAT6 phosphorylation was associated with the production of IL-13 by DC. The addition of neutralizing anti-IL-13 antibodies during maturation of DC inhibited their capability to induce IL-4 and to a lesser extend IL-5 production, but not IFN-γ production. Addition of exogenous IL-13 enhanced mainly the secretion of IL-4. Taken together, DC-derived IL-13 which is released after exposure to allergens appears to be one of the critical factors for DC, which express IL-13 receptors, to acquire the capability to induce Th2 cytokine production.

078 [Oral 023]

Inhibition of STAT1 Pathway by Suppressor of Cytokine Signaling (SOCS) 1 Prevents the IFN-γ-Induced Expression of Pro-Inflammatory Genes in Human Keratinocytes

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Contribution of keratinocytes to the pathogenesis of immune-mediated skin diseases is linked to their intrinsic capacity to respond to pro-inflammatory stimuli. IFN-γ is the cytokine to whom keratinocytes are more susceptible and respond producing a broad array of inflammatory mediators, and its inactivation would be advantageous in pathologic conditions. SOCS1 and SOCS3 are negative feedback regulators of IFN-γ signaling and are induced in many cell types by IFN-γ itself and other cytokines. We show here that SOCS1, SOCS2, SOCS3 and cytokine-inducible SH2-containing (CIS) mRNA were up-regulated by IFN-γ in normal human keratinocytes whereas only SOCS1 or SOCS1 and CIS were induced by TNF-α or IL-4, respectively. SOCS1, SOCS2, SOCS3 proteins were undetectable in healthy skin, and highly expressed in the epidermis of psoriasis and allergic contact dermatitis skin but only weakly in atopic dermatitis skin. In keratinocytes transiently transfected with SOCS1 or SOCS3 the IFN-γ-induced transactivation of an IFN-γ-responsive reporter gene was markedly inhibited. SOCS1 and SOCS3 overexpression in keratinocyte stable clones inhibited IFN-γ-induced phosphorylation of IFN-γRα, and activation of STAT1 and STAT3. Furthermore, SOCS1 and, to a lesser extent SOCS3 reduced membrane expression of ICAM-1 and HLA-DR, and IP-10, Mig and MCP-1 release by keratinocyte clones promoted by IFN-γ. SOCS1-expressing keratinocytes showed constitutive higher, but not IFN-γ-inducible, IL-8 levels compared to SOCS2 and SOCS3 clones, and were resistant to IFN-γ-mediated growth inhibition. Targeting keratinocyte SOCS1 may represent a novel therapeutic approach to IFN-γ-dependent skin diseases.

079 [Oral 024]

Autoimmunity Against Retroviral Proteins Patients with Psoriasis

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Increasing evidences suggest that Human Endogenous Retroviruses (HERVs) could participate in the pathogenesis of autoimmune diseases, including multiple sclerosis or rheumatoid arthritis. We have previously found that different families of human endogenous retroviral sequences were up regulated in the psoriatic lesion including the HERV-W, -K and –E families. In addition, we have characterised a new variant within the HERV-W family that was almost specifically expressed in psoriatic lesions. Since this family belongs to the Murine Leukemia Virus (MLV)-like group of HERVs, we have searched for antibodies against MLV proteins in the sera of psoriatic patients to assess a possible role in autoimmune processes of the disease. The screening for anti-MLV antibodies was performed in 49 psoriatic and 32 control sera by western-blot on MLV proteins obtained after collecting and centrifuging the supernatant of FlyE packaging cell line. Anti-MLV IgAGM antibodies were detected in both psoriatic and control sera. However, their detection was significantly increased in psoriatic samples compared to control (87% vs. 50%, respectively, $p = 0.001$). In addition the seroconversion to an IgG response was dramatically increased in psoriatic sera (85% vs. 10%, respectively, $p < 0.0001$). This immunoreactivity was observed against the products of both the *gag* (p30) and *env* (p15E and gp65–70) genes and the most antigenic proteins was the gp65–70. The IgM response to MLV has already been described in normal individuals and is supposed to participate to the innate immune response, however, the seroconversion to an IgG response has only been previously described in HIV positive patients. To further characterize this seroconversion, we blot-purified the antibodies reacting with the p30 MLV protein and detected a protein with a molecular weight of 50 kDa in human skin protein extracts from both normal and psoriatic reconstructed skin. These results suggest that a specific seroconversion to an IgG response against MLV proteins occurs in psoriasis and that these antibodies could cross-react against a protein present in normal human skin. The characterization of this protein would help to further understand the role of immune response against HERVs in the pathogenesis of psoriasis.

080

Human Keratinocytes Synthesize Factor H: Synthesis is Regulated by IFN-γ

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Locally synthesized complement plays an important role in host defense and inflammation at tissue and organ level. Keratinocytes have been shown to synthesize two soluble complement components, C3 and factor B. We investigated whether factor H, which controls the activities of C3 and factor B, is also synthesized and released by keratinocytes. Regulation of factor H synthesis by some inflammatory mediators was also studied. Keratinocytes were cultured for 72 h in the presence and absence of supernatant of activated peripherial mononuclear cells, IL-1α, IL-2, IL-6, TGF-β1, TNF-α or IFN-γ. Factor H was measured in culture supernatants by ELISA. Molecular species of released factor H were identified by Western blotting. Factor H transcripts were detected by RT-PCR. Regulation of factor H message was studied by a semiquantitative RT-PCR. For measurement of factor H bioactivity, cell supernatants were incubated with ^{125}I-C3b and factor I and were run on an SDS-page gel; C3b cleavage was visualized by autoradiography. Human keratinocytes constitutively released biologically active factor H. None of the above mentioned cytokines, except IFN-γ regulated its release. IFN-γ induced release of factor H was inhibited by cycloheximide. Factor H mRNA was constitutionally expressed in keratinocytes and was strongly up-regulated by IFN-γ. Keratinocytes are capable of synthesizing factor H and this synthesis is regulated by IFN-γ at pretranslational level.

081

EGF Receptor Ligands Regulate Chemokine Expression in Human Keratinocytes

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During chronic inflammatory disorders of the skin, cytokines released by infiltrating leukocytes induce keratinocytes to overexpress EGF receptor (EGFR) ligands, including transforming growth factor (TGF)-α, amphiregulin (AR), and heparin-binding EGF-like growth factor (HB-EGF), which are directly responsible of the hyperproliferative state of the involved epidermis. Little is known about the impact of EGFR ligands on TNF-α- or IFN-γ- driven keratinocyte activation. In this study we have investigated the capacity of EGFR ligands to modulate spontaneous and TNF-α- or IFN-γ-induced expression of a cluster of chemokines relevant in skin inflammation, such as GM-CSF, IL-8, MCP-1, RANTES and IP-10. Subconfluent cultures were treated with EGFR ligands alone, or associated to TNF-α or IFN-γ. A modest increase in basal GM-CSF and IL-8, and down-regulation of RANTES and IP-10 mRNA levels were evident as early as 2 h following stimulation with EGFR ligands. Moreover, EGFR ligands dose-dependently synergized with TNF-α or IFN-γ in the induction of GM-CSF and IL-8, whereas they counteracted lymphokine induction of RANTES, MCP-1, and IP-10. Parallel variations were detected at the protein level in the supernatants collected after 24 h. Pharmacological abrogation of EGFR ligand-dependent autocrine loop, performed either with EGFR blocking antibody or specific EGFR kinase antagonists, or using a broad spectrum hydroxamate inhibitor of matrix metalloproteases, significantly affected TNF-α- or IFN-γ-stimulated chemokine expression. GM-CSF and IL-8 were suppressed, whereas RANTES, IP-10 and MCP-1 were significantly up-regulated. Analysis of chemokine mRNA decay kinetics in the presence of the transcriptional blocker actinomycin D indicated that abrogation of EGFR signaling perturbed both mRNA neosynthesis and stability. Finally, EGFR kinase antagonists increased expression of IP-10 and inhibit IL-8 expression in the epidermis of skin organ cultures treated with IFN-γ. Our data suggest that cross-talk between lymphokine-induced signals and EGFR-mediated signals regulates keratinocyte activation during inflammatory processes of the skin.

082

Regulation of Expression of the Chemokine CCL27 (CTACK) During Allergic Contact Dermatitis

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Chemokines are important mediators of immune-regulated skin disorders such as allergic contact dermatitis (ACD). The differential and sequential expression of chemokines regulates the type as well as the timing of the infiltrate into the affected area. CCL27 is such a chemokine – recently it has been shown that CCL27 is inducible during the early stages of ACD and is responsible for attracting activated T cells into the skin. Whether or not this chemokine is triggered in general by early danger signals (chemical exposure) or whether it is restricted to allergens is unknown. Also how this chemokine relates to the induction, elicitation and recovery phases of ACD is largely unknown. In order to determine whether CCL27 expression is induced upon exposure to both allergen and irritant danger signals, we have patch tested human skin equivalents derived from nonsensitized individuals with allergens (nickel sulphate, potassium dichromate) and the irritant: sodium lauryl sulphate. 24 h later the culture medium was harvested and the amount of chemokine secretion was measured by ELISA. Non toxic concentrations of both allergens and irritant resulted in an increase in CCL27 secretion above its basal expression. In order to determine how this chemokine relates to the induction, elicitation and recovery phases of ACD, volunteers recruited from the contact dermatitis clinic with known contact allergy were patch tested and biopsies taken before and 4 h, 48 h, 72 h and 21 days after patch testing. *In situ* RNA hybridization showed that CCL27 mRNA was already induced 4 h after patch testing and remained increased for at least 21 days after patch testing. This indicates that increased CCL27 expression occurrs very early and remains longer than the visible effects of the patch test reaction. The late inflammatory chemokines, CXCL9, CXCL10 and CXCL11, in contrast were not induced until 48 h after patch testing and were no longer detectable 21 days after patch testing. In conclusion, CCL27 secretion is increased upon chemical exposure and is not retricted to allergenic contacts. Furthermore, CCL27 mRNA levels remain increased after the visible effects of ACD have disappeared. This may in part explain the phenomena known as "local skin memory" and flare up reactions at previous ACD sites.

083

Interferon-γ Release from Peripheral Blood Lymphocytes Following *In Vitro* Challenge with Drugs in Patients with Cutaneous Adverse Drug Reactions – Comparison with *In Vivo* Tests

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In vitro drug-induced cytokine release suggests a drug-specific immune response. The aim of the present study was to compare between *In vitro* drug-induced interferon-γ (IFN-γ) release tests and *in vivo* tests with the same drugs in a series of patients with cutanous adverse drug reactions (CADRs). Drugs taken by 36 patients with CADRs were classified into 3 categories of clinical drug suspicion: high, possible and low. Twenty-two patients taking a similar profile of drugs without adverse reactions served as controls. Following incubation of peripheral blood lymphocytes with drugs, the supernatants were collected for the detection of IFN-γ release. For each drug tested the increase in IFN-γ release was calculated. A value higher than the mean percentage increase of IFN-γ + 2SD (threshold level) measured in the controls, was determined as a positive IFN-γ-test. *In vivo* tests, including withdrawal and/or challenge tests with drugs, were evaluated in 32 patients. Positive IFN-γ release tests were recorded for 28/36 (78%) of the patients towards 56/118 (47%) of the drugs. IFN-γ release recorded for drugs taken by the patients ($72.0 \pm 90.7\%$) was higher ($p < 0.001$) than that recorded for drugs taken by the controls ($18.7 \pm 20.1\%$). The proportion of positive IFN-γ responses was associated with the degree of drug suspicion: 64%, 36%, and 28%, respectively. Positive IFN-γ responses recorded for the highly suspected drugs were increased as compared to the low suspected drugs ($p = 0.001$). Analysis of the results revealed 71.8% percent agreement between the results of *In vitro* drug-induced IFN-γ release tests and *in vivo* tests, $\kappa = 0.44$, which implies intermediate-good agreement. The results of *in vivo* tests support the diagnostic role of *in vitro* drug-induced IFN-γ release in CADRs.

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Alterations in Interferon-Gamma (IFN-γ) and Interleukin-4 (IL-4) Expression in Psoriatic Skin after Narrow Band Ultraviolet B (NB-UVB) Therapy

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There is a predominance of type 1 (i.e. IFN-γ) cytokines in diseased skin and peripheral blood of psoriasis patients. We showed that a single exposure to high dose of broad band UVB-radiation changes the type 1/type 2 (i.e. IL-4) cytokine balance of normal skin in favor of type 2 cytokines. In this study, we aimed to show whether a change in the expression of IFN-γ and IL-4 also takes place in psoriatic skin after NB-UVB therapy thrice weekly for several weeks. Skin biopsies were obtained from psoriasis patients ($n = 10$) before, at the 3rd week and at the end of NB-UVB therapy. Expression of IFN-γ and IL-4 was determined *in situ* by immunohistochemistry in frozen sections. Intracellular expression of these cytokines was measured by flow cytometry in isolated dermal $CD4^+$ and $CD8^+$ T cells. IFN-γ was abundantly expressed in all pretreatment biopsies, but this expression was decreased after the treatment with NB-UVB. Interestingly, IL-4 was also expressed, though weakly, in most of the pretreatment biopsies and this expression diminished after the NB-UVB therapy. Although there was a high interpatient variability in intracellular IFN-γ expression, dermal $CD4^+$ and $CD8^+$ T cells expressed less IFN-γ after the treatment. No clear change of intracellular IL-4 expression was observed upon NB-UVB therapy. In sum, NB-UVB therapy reduces the expression of IFN-γ and IL-4 in psoriatic skin. IFN-γ production of remaining T cells in skin after therapy diminishes upon restimulation. These could contribute to the therapeutic action of NB-UVB.

085

Pimecrolimus Demonstrates Significant Immunosuppressive Properties in a Rat Heart Transplant Model

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Pimecrolimus is an ascomycin macrolactam derivative which inhibits the proliferation of human T-cells. The aim of the study in hand was to assess the immunosuppressive properties of this compound. We employed a rat heart transplant model which has been used as a standard method to assess the *in vivo* immunosuppressive potency of substances since the introduction of cyclosporin. Thirty rats in the ACI-to-Lewis rat-strain combination underwent heterotopic heart transplantation. The rats were allocated to 3 groups with ASM 981 dosages of 0.3 mg per kg i.m. ($n = 8$), 1.0 mg per kg i.m. ($n = 8$) and 3.0 mg per kg i.m. ($n = 7$) plus a control group receiving placebo i.m. ($n = 7$). Intramuscular injections were administered once daily from the day of transplantation (Day 0) until Day 13. Allograft rejection was defined as cessation of heart beat, grafts were palpated on a daily basis. Concerning survival times, the median graft survival time (MST) in the control group was 5 days. The ASM 981 group receiving the lowest dosage (0.3 mg per kg i.m.) did not show a significantly prolonged survival time. In contrast, the 1.0 mg per kg i.m. and 3.0 mg per kg i.m. treatment groups showed a significantly increased graft survival duration (MST 14.5 days and 26 days respectively, $p < 0.01$ versus placebo for both groups). Our findings show that pimecrolimus significantly prolongs allograft survival in rat heart transplantation. We therefore conclude that pimecrolimus has substantial immunosuppressive potency and displays comparable efficacy in vivo to cyclosporin.

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Topical Tacrolimus is More Efficacious Than Pimecrolimus in Inhibiting Acute Contact Dermatitis in a Rat Model

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The immunomodulatory efficacy of tacrolimus and pimecrolimus was compared in a standardised model of oxazolone-induced skin inflammation in the rat. Three concentrations of acetone solution containing either tacrolimus (0.001%, 0.01%, 0.1%) or pimecrolimus (0.01%, 0.1%, 1.0%) were applied to the ears of rats one hour prior to challenging with oxazolone. Topical application of the solutions was repeated three hours later. The amount of swelling observed 72 h following challenge was inhibited in a dose-dependent manner by the application of either tacrolimus or pimecrolimus solution. Significant inhibition of the immunological response was observed following the application of a concentration of 0.01% tacrolimus or more and 0.1% pimecrolimus or more. Complete inhibition was achieved following application of 0.01% and 0.1% tacrolimus and 1.0% pimecrolimus. In conclusion, this model demonstrated that tacrolimus has at least a 10 times higher potential for inhibiting acute contact dermatitis compared with pimecrolimus. These preclinical data suggest that, in the clinical setting, tacrolimus ointment may show considerable superiority to pimecrolimus in the treatment of moderate to severe cases of atopic dermatitis.

087

Sera From Patients with Palmoplantar Pustulosis Induce Immunofluorescence on Endothelial Cells in Several Organs

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We previously found antibodies to nicotinic receptors in PPP sera. Forty-seven per cent of the PPP sera gave an immunofluorescence (IF) on endothelial cells in normal palmar skin from a nonsmoker, especially in the papillary dermis. On palmar skin from smokers there was also IF in the sweat duct. The staining intensity was stronger in PPP patients with nAChR ab than in those without nAChR ab both on skin from nonsmokers and smokers. The reactivity to PPP sera has now also been studied in other skin areas and in sections from normal thyroid and parathyroid gland, intestinal mucosa and pancreas as PPP patients have an increased prevalence of several autoimmune diseases. Sera was obtained from 5 PPP patients, 5 healthy nonsmoking persons and 5 patients with hand eczema. Skin biopsy specimens from healthy nonsmoking persons from scalp, fingertip, elbow, forearm and back were used together with biopsy specimens of normal thyroid and parathyroid gland, intestine and pancreas. The results showed that there was IF in the endothelium also in other skin areas but the strongest and most widespread IF pattern in the skin was found in the palmar endothelium. This difference in IF pattern might mean that palmar endothelium may have a different antigenic profile than other skin areas. PPP sera also induced an IF in endothelium in other organs, e.g. thyroid and parathyroid glands, intestinal mucosa and pancreas. Sera from healthy controls and patients with hand eczema produced no IF. The results further indicate that PPP is a systemic disease.

088

Biofilms of *Pseudomonas aeruginosa*: A New Approach for Understanding *P. aeruginosa* Skin Infections

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The success of *P. aeruginosa* (PA), an ubiquitous opportunistic human pathogen, to colonize epithelia is due to its broad spectrum of virulence factors which are induced under stress conditions. The ability of these virulent pathogens to form so-called biofilms as the consequence of density quorum-sensing is of high relevance for its pathogenity. Therefore the aim of this study was to analyse whether human keratinocytes are capable of distinguishing between highly pathogenic biofilm-forming *P. aeruginosa* and nonbiofilm forming *P. aeruginosa.* We investigated different cultures of PA incubated under stress conditions and evaluated their ability to induce the production of proinflammatory cytokines (like IL-8, IL-1β, and TNF-α) in primary human keratinocytes. The mRNA-expression in keratinocytes was measured by using RT-PCR and Realtime-PCR. We found that biofilms were formed under static growth conditions with low oxygen and starving conditions. Supernatants of these bacteria showed a highly increased induction of several proinflammatory cytokines compared to nonbiofilm bacteria. RP-HPLC of these inducing activities revealed elution in single fractions indicating a panel of different inducing bacterial molecules. A molecular characterization of these pathogen-associated molecules (PAMs) is currently in progress. In conclusion, our findings support the hypothesis that human skin keratinocytes recognise highly pathogenic biofilm-forming microorganisms such as PA via different PAMs to mount an epithelial defense reaction by producing proinflammatory cytokines to recruit professional phagocytes.

089

Investigations of mRNA Expression Patterns of Different Neuropeptide Receptors in the Lesional and Non-Lesional Skin of Patients with Psoriasis Vulgaris

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There is increasing evidence that the cutaneous neurosensory system can modulate inflammatory responses in the skin via the release of neuropeptides. Neuropeptides have been presumed to play roles in a variety of inflammatory skin diseases. The biological responses to different neuropeptides in the skin depend on the expressions of special neuropeptide receptors on the cells. In this work, the substance P receptor (NK-1R), the calcitonin gene-related peptide receptor (CGRP-1R), the melanocortin receptor (MC1-R) and two types of vasoactive intestinal polypeptide receptors (VIP-1R and VIP-2R) were investigated as concerns the mRNA level in skin biopsies from 11 patients with a hyperproliferative inflammatory skin disorder, psoriasis vulgaris, and 5 healthy persons undergoing plastic surgery. The biopsies were taken from psoriatic nonlesional ($n = 5$) and lesional areas ($n = 11$). Total RNA was isolated, and semiquantitative RT-PCR reactions were then performed by specific primer sets for different neuropeptide receptors. In the healthy control skin biopsies, only the MCR-1R was detected. In contrast, in the nonlesional psoriatic skin, NK-1R and CGRP-1R mRNAs appeared in 2 cases, each. MC-1R mRNA expression was also demonstrated in 3 of 5 nonlesional skin samples, but neither the VIP-1R nor the VIP-2R were detected. In the lesional psoriatic skin samples, NK-1R mRNA expression was demonstrated in 9 cases, CGRP-1R and MC-1R mRNAs in 8 cases and VIP-1R expression in 3 samples. Pairwise comparison of the mRNA expressions of NK-1R and CGRP-1R in nonlesional and lesional psoriatic areas of 2 patients, revealed higher mRNA levels in the lesional skin. These results support that both nonlesional and lesional psoriatic skin could be more susceptible to neuropeptide signals due to higher expression of neuropeptide receptors.

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Angiogenesis Induced by HPV16 Harboring Tumor Cells is Related Both to Generation of Angiogenic Factors and Decreased Activity of Interleukin-18

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Tumor-induced angiogenesis, i.e. new blood vessel formation within solid tumors is prerequisite for progression of solid tumors, including HPV-associated cervical carcinoma and other anogenital tumors. In addition to tumor cell-derived angiogenic factors, an important source of angiogenesis-regulating cytokines are tumor infiltrating mononuclear cells. They produce both angiogenic cytokines (e.g. IL-6, IL-8) and several inhibitors of angiogenesis- inducing IFN_s, IL-12, IL-18 and others. In previous study using the murine cutaneous model of angiogenesis we disclosed a strong angiogenic potential of tumor cells harboring HPV16 (SKv). In this study with the use of RT-PCR and ELISA we found that this potential is due to production of vascular endothelial growth factor (VEGF). We also studied whether SKv could affect production of angiogenesis-regulating factors by human peripheral blood mononuclear cells (PBMC). In order to detect biological activity of IL-18 we studied by ELISA IFNã production by PBMC of healthy donors. PBMC were in incubated with OKT-3 antibodies against CD3 molecules (0.01, 0.1, 1.0 μg per ml), with or without conditioned media from SKv cells. We found that addition of SKv conditioned media reduced significantly production of INFγ (from 1700 pg per ml to 800 pg per ml) and proliferation of PBMC. The possible mechanism of this reduction could be inactivation of IL-18 by E6 protein of HPV16, as shown in other experimental system. Since we have recently shown that IL-18 has an antiangiogenic capability, it is conceivable that this E6-dependent effect could contribute to *in vivo* stimulation of tumor angiogenesis. In addition, abrogation of IL-18 activity could result in down-regulation of NK and T cell activity, leading to escape of HPV-16 harboring tumor cells from immune surveillance. Thus we showed that the angiogenesis induced by HPV16 harboring tumor cells might be related, at least in a part, to a decreased antiangiogenic activity of interleukin-18.

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Down-Regulation of μ-Opiate Receptor and β-Endorphin in Epidermis of Atopic Dermatitis and its Correlation to INF-γ *In Vitro*

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The presence of the mu-opiate receptor system in human epidermis presents a new dimension to the research in neuro-immuno-dermatology. We have previously shown that human epidermal keratinocytes express a functional active mu-opiate receptor. Using modern confocal imaging microscopy and colocalization analysis we demonstrated that mu-opiate receptor is not only expressed in keratinocytes but also on unmyelinated peripheral nerve fibers in dermis and epidermis. Using immunohistochemistry technique with classical microscopy as well as fluorescent confocal microscopy we are able to show that in epidermis of atopic dermatitis the expressions of both mu-opiate receptor and β-endorphin are down regulated compared to normal skin. The staining was semiquantified using a digital imaging system and statistical software in confocal microscope. Furthermore we incubated human skin organ cultures for 48 h with INF-γ, a Th1 pathway cytokine, and measured the expression of mu-opiate receptor and β-endorphin by confocal microscopy. These functional experiments show a significant increase of the mu-opiate receptor and its ligand in the epidermis after exposure to INF-γ. It is well known that in atopic dermatitis the expression of INF-γ is decreased. Therefore as we expected the expression of mu-opiate receptor and its ligand is down regulated in epidermis of atopic dermatitis. Additionally we observed that in the epidermis of atopic dermatitis the terminal nerve endings are different in quantity and quality compared to normal skin. All these results suggest that the opiate receptor system in epidermis might be involved in some of the crucial symptoms associated with atopic dermatitis such as itch. Our results add new proof that the opiate receptor system play an important role in the crosstalk among the skin, the nerve system and the immune system.

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Increased Activities of D-Dopachrome Tautomerase (DDT) and Macrophage Migration Inhibitory Factor (MIF) at UVB-Induced Inflammation in the Skin

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Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine with enzymatic properties. MIF is produced by several types of cells including human keratinocytes and it is also known to be involved in several autoimmune diseases. An increased production of MIF is induced at physiological levels of glucocorticoids but MIF is also known to antagonize the immunosuppressive effects of glucocorticoids. Taken together this indicates a complex regulatory interplay between MIF and steroids. D-dopachrome tautomerase (DDT) is an enzyme that is structually related to MIF but with unknown immunologic properties. DDT and MIF both catalyze a conversion of D-dopachrome, but with different endproducts, the DHI (5,6-dihydroxyindole) for DDT activity and the DHICA (5,6-dihydroxyindole-2 carboxylic acid) for MIF activity. In this study we have focused on DDT in skin inflammation. We measured DDT and MIF in blister fluid by using the suction blister method. Prior to suction we induced experimental skin inflammation by UVB irradiation of the forearm in 10 healthy subjects. Non-irradiated sites were used as controls. The blister fluid was subsequently analysed for DHI and DHICA by HPLC and a fluorescence detector. DDT and MIF activities were demonstrated in blister fluids in all 10 subjects. All but one of these showed an increase in activity of DDT and MIF after irradiation with 3 MED UVB. The mean activities of both DDT and MIF increased approximately twofold after UVB irradiation. These differences were highly significant. The presence of DDT in epidermis was confirmed by immunohistochemical stainings in both control and irradiated human skin sections. In this study we demonstrate the presence of DDT in the skin. We also show for the first time that DDT can be related to inflammation, and the covariation between DDT and MIF strengthens this observation.

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Keratinocyte Unresponsiveness Towards Interleukin-10: Lack of Specific Binding Due to Deficient IL-10 Receptor 1 Expression

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Whereas the effects of interleukin (IL)-10 on several epithelial cell-types are well established the capability of IL-10 to target keratinocytes (KC) is still a matter of debate. The purpose of the present study was to further investigate direct effects of IL-10 on keratinocytes and to address the reason for potential IL-10 unresponsiveness using keratinocyte like cell line HaCaT as well as primary foreskin keratinocytes. Using real time RT-PCR we demonstrated that IL-10 is neither able to induce its typical early gene product suppressor of cytokine signalling (SOCS) 3 nor to modulate the interferone (IFN)-γ induced expression of SOCS 1 and 3. Although flowcytometric analyses showed binding of biotin labelled IL-10 to HaCaT cells, blocking experiments indicated that this resulted from unspecific binding. Moreover, scatchard plot analyses excluded specific binding to primary KC and HaCaT cells. Finally, real time mRNA analyses demonstrated that the absence of any specific binding results from the lack of IL-10R1 (α chain) expression, whereas the IL-10R2 (β chain) is constitutively expressed. Our data indicates that IL-10 unresponsiveness of keratinocytes could be explained by a lack of IL-10R1 expression and suggest that any IL-10 effects on these cells observed are indirectly mediated. This, however, is of considerable importance, since IL-10 is a major anti-inflammatory, immunosuppressive cytokine with impact on the cutaneous homeostasis. In particular with regard to recently proven clinical effective IL-10 therapy in psoriasis. Response to therapy is associated with normalisation of typical parameters of keratinocyte pathology.

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Pyoderma Gangrenosum Induced by Montelukast Sodium – Confirmation by Interferon-γ (IFN-γ) Release Test

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Previous reports demonstrated that pyoderma gangrenosum (PG) may be caused by drugs. *In vitro* drug-induced interferon-γ (IFN-γ) release test was previously used to confirm the role of suspected culprit drugs in patients with cutaneous adverse drug reactions. A 53-years-old male with asthma has been treated by Montelukast sodium (Singulair®) 10 mg per d. Two months later, painful ulcers appeared on the dorsal side of the arms. Additional medications were inhalation Salbutamol, inhalation Salmeterol and T. Prednisone 5 mg per d. Skin examination revealed ulcers with purpule-red, raised, undermined borders. A biopsy specimen revelaed a dense neurtrohilic infiltrate. A diagnosis of PG was made according to the clinical and histological findings. Extensive laboratory and imaging investigations did not reveal an associated sytemic disease. PG was attributed to drug intake, with Montelukast sodium being the suspected culprit drug. All systemic medications were withdrawn and the patient was treated by prednisone 60 mg per d with gradual resolution. Patch tests with Montelukast sodium were borderline positive in concentrations 0.01% and 0.1%. In order to confirm the role of Montelukast sodium as the culprit drug, IFN-γ release test was performed. Briefly, following incubation of peripheral blood lymphocytes with drugs, the supernatants were collected for the detection of IFN-γ release. The increase in IFN-γ release was calculated for each drug tested. A positive IFN-γ-test was defined as a value higher than the mean percentage increase of IFN-γ + 2SD (threshold level) measured in controls taking the same drug porfile. IFN-γ release for Montelukast sodium (the suspected culprit drug) was 53.3% as compared to a control threshold of 44.8%. IFN-γ release for prednisone (which was not suspected as a culprit drug) was not increased as compared to controls. In conclusion, in the patient described, the results of IFN-γ release test support the role of Montelukast sodium as the culprit drug in the induction of PG.

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Agonists of Proteinase-Activated Receptor-2 Stimulate Activation of NFκB and Up-Regulation of ICAM-1 in Human Keratinocytes

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It is well known that serine proteases are generated in the skin and are involved in several biological processes such as growth, differentiation and inflammation. Proteinase-activated receptor-2 (PAR-2) belongs to a new G protein-coupled receptor subfamily activated by various serine proteases. PAR-2 has been demonstrated to play a role during cutaneous inflammation *in vivo*. PAR-2 is expressed by human keratinocytes (KTC) and regulates inflammatory responses, proliferation and differentiation in these cells. However, the underlying mechanisms of PAR-2 activation in KTC are still incomplete. Northern blot analysis revealed PAR2-RNA in primary KTC and endothelial cells. Moreover, increased RNA levels were detected in lesional skin of patients with atopic dermatitis. Ca-mobilization studies demonstrated that PAR-2 is functional in human KTC induced by PAR2 agonist tryptase. PAR-2 agonists induce up-regulation of ICAM-1 RNA by RT PCR. Electrophoretic mobility shift assays and morphological transduction studies revealed PAR2-induced activation and translocation of NFκB in KTC and also in microvascular endothelial cells (HDMEC) with a maximum after 1 h. Use of NFκB inhibitor BAY7082 prevented up-regulation of the cell adhesion molecule ICAM-1 in KTC. In conclusion, PAR-2 induces NFκB activation and up-regulation of cell adhesion molecules such as ICAM-1. Thus, PAR-2 may play an important regulatory role on human KTC function under physiological and pathophysiological conditions.

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Evidence for a Proinflammatory Role of PAR-2 During Cutaneous Inflammation *In Vivo*

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Contact dermatitis (CD) is a frequent dermatological disease with a high socio-economical impact characterized by acute to chronic inflammation of the skin often leading to therapy-resistent excema. Proteinase-activated receptor-2 (PAR-2), a G protein-coupled receptor for certain serine proteases, is localized on keratinocytes, endothelial cells and nerve fibers, and has been demonstrated to play a role during inflammation of several tissues. However, the precise role of PAR-2 and the underlying mechanism of PAR2-induced regulation of inflammation is still fragmentary. Therefore, we were interested in whether or not PAR-2 is involved in cutaneous inflammation using a model of experimentally induced allergic (ACD) and irritant (ICD) contact dermatitis. In wild-type ($PAR2^{+/+}$) mice, PAR2 agonists induced an increased intradermal edema and enhanced plasma extravasation with a maximum between 3 and 24 h. These inflammatory responses were significantly diminished in PAR2-deficient ($PAR2^{-/-}$) mice and controls (vehicle). Morphological analysis revealed a dramatic increase of spongiosis and intradermal edema along with enhanced infiltration of neutrophils and monocytes in $PAR2^{+/+}$ mice as compared to $PAR2^{-/-}$ mice. Interestingly, nitric oxide (NOS)-inhibitors significantly diminished these effects indicating a role of NO in PAR2 -induced inflammatory responses of the skin. Functional studies at the RNA- and protein level further revealed PAR2-induced up-regulation of the cell adhesion molecules ICAM-1 and E-selectin by dermal microvascular endothelial cells during inflammation suggesting that PAR-2 directly regulates cell adhesion molecule function during skin inflammation. PAR2 agonists also stimulated up-regulation of mediators involved in cutaneous inflammatory responses such as IL-6 and NO in murine and human (dermal) endothelial cells. Together, these results strongly suggest a proinflammatory role of PAR-2 during CD and probably other inflammatory dermatoses, especially during the early phase characterized by edema, plasma extravasation and recruitment of inflammatory cells to the site of inflammation. Thus, PAR-2 antagonists may be helpful tools for the treatment of inflammatory skin disorders such as contact dermatitis and atopic eczema.

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Tumour Necrosis Factor-α Induced Up-Regulation of Elafin and Antileukoprotease Enhances the Skin Barrier

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In normal human epidermis, there is a balance of corneodesmosome proteolysis and inhibition of the proteases involved in order to maintain stratum corneum thickness and skin barrier function. This balance is known to be disrupted in diseases such as psoriasis whose symptoms include a thickening of the stratum corneum. Proteolysis of corneodesmosomal proteins is thought to be involved in the maturation of corneodesmosomes, and the cleavage of proteins such as corneodesmosin, desmocollin, desmoglein, plakoglobin and desmoplakin is thought to be a prerequisite for desquamation. Recently, corneodesmosin and plakoglobin have been shown to be processed by stratum corneum chymotryptic and tryptic enzymes (SCCE and SCTE) *In vitro*. Antileukoprotease (SLPI) and elafin are two serine protease inhibitors which serve to inhibit SCCE and SCTE, suggesting that SLPI and elafin are involved in the regulation of the proteolysis of corneodesmosomal proteins. Tumour Necrosis Factor-α (TNF-α) is known to up-regulate the expression of elafin. In addition, TNF-α, SLPI and elafin expression are up-regulated in epidermis from psoriasis patients. Here we used semiquantitative RT-PCR to measure the expression of elafin and SLPI in human reconstituted epidermis and psoriatic skin. Epidermal equivalents were treated with 2.5 ng per ml TNF-α with or without 10 μg per ml monoclonal anti-TNF-α antibody or medium only for 20 h at 37°C. Total RNA was extracted from treated and untreated reconstituted skin and from psoriatic epidermis. RT-PCR was performed on serial RNA dilutions. Both elafin and SLPI were shown to be up-regulated in skin treated with TNF-α. This effect was completely inhibited when anti-TNF-α was added to the culture medium. We confirm the up-regulation of elafin expression by TNF-α and show that this effect is specifically due to TNF-α by the loss of the effect following neutralisation with a monoclonal antibody. We also report that TNF-α up-regulates SLPI expression in a similar manner to elafin. In conclusion, in psoriasis TNF-α promotes inflammation by recruiting T-cells but also impairs desquamation by up-regulating SLPI and elafin thus decreasing the proteolysis of corneodesmosomal proteins. Therefore, anti-TNF-α antibody treatments for psoriasis are effective due to their anti-inflammatory effect but also via the inhibition of the enhancement of the skin barrier caused by TNF-α.

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Implication of Epstein-Barr Virus in Sézary Syndrome

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Cutaneous T cell lymphomas (CTCL) are characterized by the infiltration of the skin by activated T lymphocytes of the CD2+ CD3+ CD4+ CD45RO+ DR+ phenotype. The aetiology of this pathology remains still unknown. Some viruses such as EBV and HTLV-1 have been investigated for years to play a role in this disease, but the results remain still debated. The aim of our study was to precise the potential role of EBV in CTCL knowing that EBV DNA has previously been identified in cutaneous lesions of CTCL. This work was performed with blood samples of patients whose Sézary Syndrome has been confirmed by histology, immunochemistry and clonal rearrangement and with more than 50% of circulating Sézary cells. We tried to determine if an EBV specific T lymphocyte response towards the autologous B-EBV line could be detected in these patients which could play a trole in the chronic stimulation of T lymphocytes. This response was measured by proliferation test, cytotoxicity and production of cytokines TNF-α, IL-4 and IFN-γ. Effectors cells were clones of PBL, PBL stimulated and grown with IL-2 and IL-7, or T lymphocytes extracted from lesional skin of the patients and grown with either IL-2 and IL-7, or IL-2 and IL-15 or IL-7 and IL-15. Concurrently, we have investigated the PBL non sorted or positively Vβ cell-sorted, for the presence of mRNA of EBV, EBER-1 and BZLF-1. We showed neither a cytotoxic activity nor a production of cytokines, specific of the T lymphocytes towards the autologous B-EBV line.EBV DNA not detected in the patients' PBL. However, we observed that T lymphocytes populations proliferated strongly in the presence of the autologous B-EBV cell line. These results suggest that EBV could play a role in the development of CTCL, perhaps as an antigenic stimulus by maintaining the proliferation of the T lymphocyte population in the lesional skin. Moreover, since B-EBV cell lines do not express the whole proteins of the virus, it would be of interest to study T lymphocytes populations towards Cos cells transfected with one of the 18 cDNA coding for the EBV proteins.

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The Serum Concentration of IP-10 in Inflammatory Skin Diseases

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In the past, chemokines have been shown to be involved in homeostatic regulation as well as in inflammatory tissue reactions. They are able to focus and amplify the cellular response by activation and attraction of both resident and migratory cells. The chemokine IP10 (10 kDa peptide, induced by IFN γ) has been well characterized before definition of the chemokine family. It seems to play an important role in chronic inflammation, as indicated by its T-cell stimulating properties and local involvement in psoriatic tissue reactions. The aim of this study was to determine whether serum levels of IP-10 reflect such processes. A specific and sensitive IP-10 ELISA could be established by using two monoclonal antibodies raised in the laboratory. IP-10 serum levels were evaluated of patients with psoriasis vulgaris (n = 40), generalized eczema (n = 10), erysipelas (n = 12), herpes zoster (n = 10) and healthy volunteers (n = 40) In the healthy control group IP-10 was below the detection limit whereas in all disease groups elevated serum IP-10 levels were found. Highest IP-10 concentrations were found in erysipelas (mean 2,1 ng per ml) and psoriasis (mean 0,58 ng per ml). In psoriasis, data did neither correlate with disease activity nor with therapeutic response. Therefore, IP-10 seems to be a marker for inflammatory skin diseases but is rather unspecific concerning their activity and pathogenesis. Accordingly, release of chemokines into circulation is different in inflammatory skin disorders and perhaps reflects differential roles in systemic inflammatory processes.

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Activation/Inactivation of Classical Pathway of Complement in Nonlesional SLE Skin

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Lupus erythematosus (LE) is characterized by the deposition of immunoreactants at the basement membrane zone of the skin (the lupus band). The infrequent finding of MAC in nonlesional skins with positive lupus band raised two questions: Does complement activation occurs in the nonlesional SLE skin? If it is activated, why there is no skin damage? A probable answer is that complement activation in nonlesional SLE skin might be incomplete. We postulated that in the nonlesional SLE skin, IgG binds C1q and activates the classical pathway of complement and, if the MAC is formed, it is inactivated by the induction of protectin (CD59). Non-lesional skin from 5 DLE and 20 SLE patients were investigated. Sections of skin lesions from 10 patients with DLE, 5 lichen planus, and normal skin were used as controls. C4d, the degradation product of C4 was used as a marker of complement activation. Cryostat sections (7 μm were incubated with the mouse Moab C4d (Quidel, France), washed with PBS and then incubated with FITC-conjugated rabbit antimouse antibody. Other skin sections were used to identify CD59 expression by the rat Moab MCA715 (Serotec, UK). Skin sections were incubated with the MCA715 antibody, washed with PBS and then, incubated with FITC-conjugated goat antirat antibodies (Dako, Denmark). The results showed C4d deposition in 18/20 (90%) of nonlesional SLE skin. C4d was not detected in the nonlesional skin from the 5 DLE patients. The nonlesional SLE skin showed strong expression of CD59 in 16/20 SLE patients. The 5 nonlesional DLE skin demonstrated CD59 staining comparable to normal skin. CD59 expression was low in 7/10 DLE lesions. The lichen planus samples showed normal expression of CD59 suggesting that the low CD59 expression in DLE is not related to the inflammatory component. In conclusion, the classical complement pathway is activated and the expression of the complement regulatory protein CD59 is up-regulated in the nonlesional SLE skin. This finding suggests that the protection of skin during the presence of immunoreactants in SLE skin might be facilitated by the induction of complement regulatory protein CD59. This could explain the link between the lupus band and the pathogenesis of cutaneous lupus.

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Hepatocyte Growth Factor/Scatter Factor (HGF/SF) Up-Regulates β1 Integrin Expression on Melanoma Cell Surface

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HGF/SF is known to have a motogenic effect on tumor cells leading to tumor cell invasion into connective tissue and distant metastasis. Despite the putative involvement of HGF/SF in the progression of malignant melanoma, the mechanism responsible for the invasion and metastasis process has largely been unexplored. In this study, the possible role of HGF/SF in melanoma progression was assessed by analyzing the regulatory effect of HGF/SF on integrin expression and motile activity in melanoma cells. HGF/SF dose- and time-dependently augmented β1 integrin expression in melanoma cell lines expressing c-Met but failed to regulate that in a c-Met negative melanoma cell line. On the other hand, the expression of α1-α6 and β3 integrin subunits was not modulated by HGF/SF at any concentration and in any stimulation period. In accordance to the increment of β1 integrin expression, HGF/SF stimulation enhanced attatchment of c-Met positive melanoma cell lines to type I, type III, type IV collagen, fibronectin and laminin. Furthermore, HGF/SF effect on β1 integrin expression enhanced both the invasiveness of melanoma cells through Matrigel composed of basement membrane components and the migration activity of melanoma cells on extracellular matrix-coated substrate. The findings suggest the important role of HGF/SF in invasive and metastatic potential of melanoma cells.

102

Control of Caspase-1 Activity by Caspase Recruitment Domain (CARD) Proteins in Human Keratinocytes

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The cysteine protease caspase-1 (formerly interleukin (IL)-1β converting enzyme/ICE) cleaves the precursors of the cytokines IL-1β and IL-18 into their bio-active, mature forms. We have previously demonstrated constitutive expression of proIL-1β and proIL-18 by human keratinocytes and were interested to determine whether the absence of processing was the result of a deficiency in caspase-1 production or more complex inhibition. Total RNA and protein extracts were prepared from primary human keratinocytes and assessed for caspase-1 production. Constitutive caspase-1 transcription was demonstrated by RT-PCR and Western blotting revealed that caspase-1 was consistently present in the inactive 45 kDa form. Functional caspase-1 activity was confirmed in lysates from the monocytic cell line THP-1, by the presence of processed IL-18 whereas only precursor was seen in keratinocytes. Caspase-1 is synthesised as a zymogen which oligomerises and autoprocesses upon receipt of an appropriate signal. A series of novel proteins which specifically bind caspase-1 and facilitate oligomerisation have been described over the last 2 years. Like caspase-1, they all possess a CARD through which association occurs. Two molecules, RICK and IPAF/CARD-12 induce cleavage of caspase-1 on binding, whereas the CARD proteins ICEBERG, COP/pseudoICE and CARD-8/CARDINAL all inhibit caspase-1 processing. RT-PCR analysis of human keratinocytes demonstrated strong constitutive expression of all three antagonistic CARD proteins and RICK, whereas IPAF was weakly transcribed. Further, ICEBERG mRNA production was significantly higher in keratinocytes than any other cell type tested, suggestive of a net anticleavage phenotype in these cells. These results indicate that activation of caspase-1 in keratinocytes requires a complex regulatory mechanism involving at least 5 specific CARD proteins which may influence the activity of pro-inflammatory cytokines such as IL-1β in cutaneous inflammation.

103

Interleukin-10 Therapy Reduces the Incidence of Relapse and Prolongs the Relapse-Free Interval in Psoriasis

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The ability of interleukin-10 therapy to reduce the severity of exacerbated psoriasis has been demonstrated recently. Considering the immunobiological properties of this cytokine we investigated the effects of long-term interleukin-10 application on the immune system and duration of psoriasis remission. We performed a placebo-controlled, double blind, phase II trial using interleukin-10 in patients with chronic plaque psoriasis in remission. Patients received subcutaneous injections with either interleukin-10 (10 μg per kg body weight; n = 7) or placebo (n = 10) 3 times per week until relapse or study termination after 4 month. The treatment was well tolerated. In the placebo group almost all patients (90%) showed a relapse during the observation period. In contrast to this, only 2 out of 7 patients (28.6%) relapsed in the interleukin-10-treated group. Kaplan Meier analysis revealed a significantly lower relapse incidence in the interleukin-10 than in the placebo group (p = 0.02). The mean relapse-free interval time was 101.6 ± 12.6 days in the interleukin-10 group in comparison to 66.4 ± 10.4 days in the placebo group. Immunological activity of interleukin-10 application was indicated by an increase in soluble interleukin-2 receptor plasma levels and higher *ex vivo* interleukin-4 secretion capacities. Remarkably, a significant negative correlation was demonstrated between the IL-4 secretion capacity and Psoriasis Area and Severity Index score ($r = 0.36$, $p < 0.01$). Our data suggest that interleukin-10 therapy is immunological effective, decreases the incidence of relapse and prolongs the disease-free interval in psoriasis. Its value should be further determined in larger trials and for the prevention of re-exacerbation of other inflammatory disorders with a similar immunological profile.

104

The Expression and Function of Pattern Recognition Receptors in Vaginal Epithelial Cells

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Epithelial cells of the vagina are important in providing protection against various pathogenic microorganisms, e.g. *Candida albicans*. It is known that vaginal epithelial cells produce antimicrobial peptides, which mediate the killing of pathogenic fungi. Toll-like receptors (TLRs) are known to be important in mediating mechanisms of innate host defence. We aimed to explore whether Toll-like receptors and activation of NF-κB could be responsible for the protection against pathogenic microorganisms by vaginal epithelial cells. RT-PCR and Western-blot analysis were used to examine the expressions of the two main pattern recognition receptors (TLR2 and TLR4) in immortalized vaginal epithelial cells (PK cells). PK cells were transfected with NF-κB luciferase construction and the activation of NF-κB was examined by measuring luciferase activity in the cells induced with *Candida albicans* or lipopolisaccharide (LPS). Real-Time RT-PCR was used to examine the expressions of TLRs and interleukin-8 (IL-8) after incubation with *Candida albicans* and LPS. ELISA was used for detection of cytokines produced by PK cells. We found that unstimulated cultured vaginal epithelial cells expressed TLR2 and TLR4 receptors. The expressions of TLR2 and TLR4 were up-regulated by induction with *Candida albicans* or LPS. *Candida albicans* and LPS induced the expression of IL-8 gene and the production and secretion of IL-8. In our work, we give the first evidence that vaginal epithelial cells express toll-TLR2 and TLR4 and microbial compounds activate NF-κB, which may be involved in pathogen-mediated intracellular signalling. Our findings stress the importance of the role of vaginal epithelial cells as participants of innate immunity.

105

Cellular Infiltrate and Related Cytokines of Autologous Serum Induced Wheals in Patients with Chronic Idiopathic Urticaria

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Chronic autoimmune urticaria identifies a group of patients with chronic idiopatic urticaria (CIU) and circulating autoantibodies (IgG) against the high-affinity IgE receptor and/or against IgE. These autoantibodies have the capability to provoke *in vivo* a wheal and flare response in a considerable group of patients by intradermal injection of autologous serum (autologous serum skin test, ASST). Serial biopsies of autologous serum induced wheals were performed at different times (10′, 30′, 60′, 24 h, 48 h) from the onset of the wheals, in order to assess the evolution of the cellular infiltrate and to clarify the possible pathogenesis of this skin-disorder. The study was performed on induced wheals of 27 patients (13M, 14F; median age: 43,9 years; range: 22–69) compared with the skin of 4 healthy donors. We performed an immunohistochemical analysis of the infiltrating cells (mast cells, T lymphocytes, neutrophils, eosinophils), related cytokines (IL4, IL5, IL8, IFN-γ), and chemokine-receptors (CCR3, CXCR3) as well as integrins (ICAM-1, V-CAM, ELAM-1). In the early wheals (10′) we documented an increased number of T lymphocytes and eosinophils when compared to healthy skin. Perivascular neutrophils and eosinophils showed a tendency to increase from 10 min to 1 h, as well as T lymphocytes did. At 60 min neutrophils (80 ± 57,6) rappresented the main cellular population. Activated eosinophils were increased at 24 h (EG2 19,3 ± 5) and 48 h (EG2 28 ± 2,8) whereas neutrophils decreased and mast cells did not show a significant variation. Referring to cytokines IFN-γ showed a focal dermal staining that remained almost constant at different times; IL-4 was increasing within the first hour and then decreased while IL-5 was mainly appreciated at 30′ and 60′. Our analysis in induced wheals demonstrated the presence of a T cell population with a prevalent Th2-like pattern, as we already observed in spontaneous wheals.

106 [Oral 005]

Transduction of Murine Dendritic Cells by Antigen-Encoding Lentivirus Vectors Permits Antigen Processing and MHC Class I-Dependent Presentation

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Because antigen-presenting dendritic cells (DC) play a major role in the polarization of T cells, including Th2 cells involved in allergy, strategies to modify DC genetically are required. The purpose of this investigation was to transduce murine bone marrow-derived DC with lentiviral vectors encoding antigen and to demonstrate antigen processing by the endogenous pathway and MHC class I dependant presentation. Bone marrow leukocytes were incubated with antigen-encoding lentiviral constructs and cultured with GM-CSF, IL-4 and Flt-3 ligand. The capacity of the resulting DC to express, process and present antigen was examined *In vitro*. An average of 40% of DC expressed antigen after one week of culture when antigen was green fluorescent protein encoded by the lentiviral vector construct. To demonstrate that transduced antigen can be presented by DC on MHC class I, we chose the lymphocytic choriomeningitis virus glycoprotein (gp) as a model antigen that can be recognized by CD8 T cells from transgenic mice expressing an MHC class I-restricted T cell receptor specific for the epitope of positions 33–41 from gp. DC transduced with lentiviral construct encoding gp and matured with LPS activated transgenic T cells in an antigen-specific fashion. Using transporter-associated with antigen presentation (TAP)-deficient mice, we showed that presentation of the gp33–41 epitope is TAP-dependent, confirming processing of gp by the endogenous pathway. These results demonstrate that CD8 T cells can recognize MHC class I epitopes processed from antigen by DC transduced with lentiviral vectors. Targeting of DC and antigen presentation to CD8 T cells could be exploited for immunotherapy because allergen-specific CD8 T cells have been shown to be suppressive in IgE-dependent allergy models.

107 [Oral 045]

Polymorphism A(–596)G of Interleukin-6 Gene is Associated with Cutaneous T-Cell Lymphoma

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Skin lesions from patients with mycosis fungoides contain high levels of interleukin-6. Thus, the aim of the study was to investigate the possible effects of genetic polymorphism A(– 596)G of interleukin-6 in patients with cutaneous T-cell lymphoma (CTCL). We performed a case-control study to compare genotype distributions and/or allelic frequencies between 61 Czech patients with CTCL and 61 control subjects matched for aged and sex. In all of these subjects, polymorphism A→G of promoter of interleukin-6 gene at position –596 was determined by PCR and restriction analysis. There was a highly significant difference of distribution of genotypes between the patients and the controls (p = 0.001). When the male and female patients group were compared to controls separately, this difference kept the statistical significance (p = 0.04 for males, p = 0.03 for females). Further analysis showed a significantly higher relative risk of the heterozygote (AG) genotype in patients (OR = 3.94, 95% confidential interval 1.84–8.45, p = 0.0003). This genotype is associated with higher relative risk for T-lymphoma both in males (OR = 3.23, 95% confidential interval 1.24–8.44, p = 0.01) and in females (OR = 5.20, 95% confidential interval 1.41–19.18, p = 0.01), where the strongest association has been investigated. We conclude that A(–596)G polymorphism of interleukin-6 gene is associated with cutaneous T-cell lymphoma. Heterozygote genotype carries significantly higher relative risk for cutaneous T-cell lymphoma, especially in women.

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Resistance to Fas-Mediated Lymphocyte Cell Death in Sezary Syndrome: Partial Reversal by Interferons

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Membrane bound Fas ligand (FasL, Apo-1 L, CD95L) induces rapid apoptosis of Fas (CD95)– sensitive cells upon interaction with Fas, and is an important effector molecule of cytolytic T lymphocytes. Cutaneous lymphomas including Sezary syndrome (SSy) are responsive to therapies that stimulate cellular immune responses but tend eventually to escape immune destruction. We investigated Fas expression and function in 2 SSy cell lines (Myla, SeAx), as well as in peripheral blood mononuclear cells (PBMC) from 11 SSy patients and 4 healthy controls. Fas expression was found to be virtually absent in SeAx cells, but conserved in Myla cells. Analysis of sensitivity to recombinant human FasL *In vitro* revealed that loss of Fas surface expression correlated with resistance to FasL-mediated cell death, SeAx cells being resistant as opposed to Myla cells. In SSy patients, when compared to healthy controls, Fas surface expression was reduced on CD4+ lymphocytes in 5/11 cases (45%). When susceptibility to FasL-mediated apoptosis was tested in PBMC from these patients 6/11 (55%) were significantly more resistant to FasL as compared to healthy controls. To determine if susceptibility to FasL-mediated apoptosis can be enhanced, we tested the effect of interferon α (IFNα) and γ (IFNγ) on Fas expression and signaling function in PBMC from 2 healthy controls and 2 SSy patients with low lymphocyte Fas expression. IFNα and IFNγ induced a significant increase in Fas expression in both healthy control and SSy CD4+ cells. Under these conditions, an increase in sensitivity to FasL-mediated apoptosis was also observed. This data shows that resistance to Fas-mediated apoptosis is common in CD4+ lymphocytes of SSy patients. It also suggests IFNα and IFNγ can sensitize SSy patients CD4+ cells to FasL, and has important implications for the use of interferon-related therapy of Sezary syndrome.

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Detection of Clonal T Cell Receptor Rearrangements Correlates with the Occurrence of Clonal Chromosomal Aberrations in Cutaneous T Cell Lymphomas

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Detection of clonal T cell receptor (TCR) gene rearrangement by PCR and subsequent high-resolution electrophoresis has become an important tool in the diagnosis of cutaneous T cell lymphomas (CTCL). However, TCR clonality indicates a quantitative alteration of T cells and cannot demonstrate malignancy since it is also found in a substantial portion of certainly benign dermatoses as lichen sclerosus et atrophicus, lichen ruber, and pityriasis lichenoides. We therefore asked, whether TCR clonality in CTCL can be correlated with the occurrence of clonal chromosomal aberrations (chromosomal clonality) indicating qualitative and functional alteration of the affected cells. Forty samples derived from skin, blood or lymph nodes from 20 patients suffering from large plaque psoriasis (LPP, n = 3), mycosis fungoides (MF, n = 12), or Sézary's syndrome (SS, n = 5) were simultaneously and blindly investigated for TCR and chromosomal clonality. To determine TCR clonality, fluorescence fragment analysis and TCR sequencing was applied, whereas chromosomal clonality was analysed by G-banding, comparative genomic hybridisation and interphase or 24-color *in situ* hybridisation methods. Twenty-eight samples gave results with both approaches, which corresponded in 22 (18 clonal, 4 nonclonal) but diverged in 6 (4 clonal by TCR analysis only, 2 clonal by chromosome analysis only). Identity of the cells bearing clonal TCR rearrangement and clonal chromosomal aberration was demonstrated by investigation of single cells, which were picked according to their *in situ* hybridisation pattern and subsequently analysed by sequencing the TCR rearrangement. We here, for the first time, show that TCR clonality correlates with the occurrence of clonal chromosomal aberrations in CTCL. Divergence of the results in 6 of our cases may be due to sensitivity problems of G-banding, comparative genomic hybridisation and interphase hybridisation techniques as well as nonamplification of the clonal TCR rearrangement. In conclusion, detection of a TCR clone indicates a qualitative T cell alteration, at least in CTCL.

110 [Oral 009]

Inhibiting Tumor-Angiogenesis by Specific T Cell Immune Responses

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The transgenic mouse RIP1-Tag2 is a model of multistage carcinogenesis. In these mice SV40 T antigen (Tag) is expressed only in insulin-producing b cells of the pancreas, causing about 2% of the islets to develope into adenomas and carcinomas. At 14 weeks mice die of hypoglycemia. Based on recent data obtained with transplanted tumors, we analyzed the capacities of Tag-specific CD4+ Th1 cells in the therapy of this endogenous, MHC class II-negative tumor. Tag-specific Th1 cells were generated by stimulating CD4+ cells from T cell receptor (TCR) transgenic C3H mice bearing a TCR specific for a Tag peptide. Starting at 7 weeks, the time of early adenoma development, RIP1-Tag2 mice recieved 10^7 Th1 cells weekly. We followed, blood glucose, histology and angiogenesis. Therapy with Tag-specific Th1 cells prolonged life two-fold. Surprisingly, Th1 therapy did not induce diabetes even though all islet cellls express Tag. At 13 weeks histology showed large, vascularized insulinomas in sham-treated mice. In sharp contrast, Th1-treated animals had only small poorly vascularized adenomas. Depletion of CD8+ cells influenced tumor development neither in sham-treated nor in Th1-treated RIP1-Tag2 mice. Tumor-angiogenesis is closely associated with the expression of the $\alpha v\beta 3$ integrin on endothelia. To quantify angiogenesis, we determiend uptake of radiolabeled cyclic Gluco-RGD that selectivly binds to $\alpha v\beta 3$. In untreated mice Gluco-RGD uptake started to increase at 7 weeks of age and reached 8-fold higher levels at 8 weeks. In sharp contrast, over the same period Gluco-RGD uptake remained at back ground levels in Th1-treated mice. Again, inhibition of angiogenesis was not inhibited by CD8 depletion. Thus, adoptive transfer of Tag-specific Th1 cells was highly efficient in tumor therapy and did not cause detectable side-effects. Surprisingly, these Tag-specific Th1-cells did not destroy the cells bearing the tumor-associated antigen but delayed growth and development of Rip-Tag2 tumors by arresting tumor anngiogenesis. The data provided here unravel a novel mode of interaction between tumor and specific immune responses.

111 [Oral 011]

Cutaneous Lymphocyte Associated Antigen (CLA)$^+$CD25$^+$CD4$^+$ T Lymphocytes Regulate Nickel-Specific T Cell Responses

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Regulatory CD4$^+$ T cells involved in the maintenance of peripheral tolerance have been recently identified. These cells constitutively express the CD25 antigen, are anergic *In vitro* upon triggering of the TCR, and suppress T lymphocyte activation through a mechanism requiring cell-to-cell contact. We found that approximately 20% of the peripheral blood CD4$^+$CD25$^+$ T cells express the cutaneous lymphocyte-associated antigen, a marker for skin-homing T lymphocytes. Here, we evaluate whether CD4$^+$CD25$^+$ T cells obtained from the peripheral blood of individuals with nickel allergy and from healthy controls could affect nickel-specific T cell responses *In vitro*. CD4$^+$CD25$^-$ T cells from both allergic (n°6) and non allergic (n°6) donors strongly proliferate to nickel. Purified CLA$^+$CD4$^+$CD25$^+$ T cells from allergic individuals proliferate and secrete IFN-γ and IL-10 when exposed to the metal, but fail to suppress nickel-specific T cell responses when cocultured with CD4$^+$CD25$^-$ and CD8$^+$ autologous T lymphocytes in the presence of antigen presenting cells. In contrast, CLA$^+$CD4$^+$CD25$^+$ T cells from healthy, non allergic individuals show a limited or absent proliferation to nickel, but up-regulate the CTLA-4 antigen, and dose-dependently suppress (50–85%) the proliferation and the cytokine release of CD4$^+$CD25$^-$ T cell population in the presence of the metal. The lack of suppressive capability of CD4$^+$CD25$^+$ T cells from allergic donors appear limited to nickel-specific T cell responses, since both allergic and non allergic CD4$^+$CD25$^+$ cells strongly suppress allogeneic CD4$^+$ T cell activation. These results indicate that CD4$^+$CD25$^+$ cells can regulate peripheral immune responses to haptens, and they may have a role in preventing the development of undesired skin allergies.

112 [Oral 012]

Proteasome Inhibitor PS519 Reduces Delayed-Type Hypersensitivity Response via Inhibition of Carbohydrate Determinant Synthesis of Cutaneous Lymphocyte-Associated Antigen (CLA) and T-Cell Rolling

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The first step in the process of T-cell recruitment to skin is mediated through the interaction of cutaneous lymphocyte-associated antigen (CLA) and its ligand E-selectin, a central prerequisite for delayed-type hypersensitivity (DTH) responses. In the set of glycosyltransferases involved in the synthesis of CLA α1,3-fucosyltransferase VII (FucT-VII) and β1,4-galactosyltransferase I (β4GalT-I) are believed to be mainly responsible for the regulation of its expression on T-cells. Since at least FucT-VII has a putative NF-κB binding site we addressed the question if the proteasome inhibitor PS519 suppresses CLA expression, T-cell rolling and DTH. PBMCs from healthy volunteers were stimulated with the superantigen TSST-1, a known inducer of CLA expression on T-cells, in the absence or presence of nontoxic concentrations of PS519 (1–10 μg per ml). PS519 blocked the activation of NF-κB as visualized by EMSA and markedly reduced the expression of FucT-VII as determined by RT-PCR. A parallel quantitative determination of the backbone (PSGL-1) and the set of glycosyltransferases involved in the synthesis of CLA (C2GnT, β4GalT-I, FucT-IV, FucT-VII) by real-time PCR (ABIPrism7700) revealed a decrease of FucTVII, C2GnT and β4GalT-I upon PS519 treatment (5 μg per ml). Concomitantly, CLA showed a constant inhibition by PS519 up to 7 days (38,8 ± 13,6 vs. 5,8 ± 3,1, n = 5, mean ± SD) as did CD15s expression and E-selectin binding. Using intravital microscopy with fluorescently labeled human T-cells injected retrogradely into the right carotid artery of mice we observed a significantly decreased *in vivo* rolling of PS-519 treated T-cells in the left ear postcapillary venules (16,9 ± 5,7 vs. 1,8 ± 2,6%, n = 3/13, $p < 0.001$). The functional relevance of these findings could be further corroborated in a DTH model were a significant decrease of ear swelling could be observed in 1 mg/KGbw i.v. treated BALB/c mice (161 ± 37 vs. 92 ± 46 μm, n = 130, mean ± SD). We conclude that the inhibition of NF-κB through PS519 reduces the expression of FucT-VII in T-cells leading to decreased CLA expression with less rolling in skin vessels and reduced DTH response *in vivo*.

113 [Oral 015]

Abrogating the Pathogenicity of Autoreactive T Cells with Anti-Psoriatic Agents

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Psoriasis is an inflammatory T cell mediated autoimmune disease dominated by interferon γ (IFN-γ) producing type 1 T cells infiltrating skin lesions. Therapy with fumaric acid esters (FAE) is effective for psoriasis in a large number of patients. In order to understand the underlying mechanisms we compared the *In vitro* and *in vivo* effects of FAE on human and mouse T cells. Eighteen patients with chronic psoriasis were treated with FAE according to a standard regimen. The dose of FAE was increased individually, serum and PBMC were analyzed at regular intervals. Leukocyte counts and relative distribution of subsets remained largely unaffected, except for an increase of eosinophils. Serum IgE levels were not influenced. In humans, intracellular cytokine analysis of freshly isolated T cells showed a significant suppression of the IFN-γ/Interleukin 4 (IL-4) ratio of CD4$^+$ T cells, starting at about 3 weeks of treatment. Importantly, suppression of the IFN-γ/IL-4 ratio in peripheral CD4$^+$ T cells was accompanied by a marked decrease of PASI (> 70%). To determine whether this deviation of Th1 into Th2 responses was causally related to the improvement, we investigated the effect of FAE on autoreactive Th1 cells to transfer autoimmune diseases in mice with experimental autoimmune encephalitis (EAE). Feeding FAE significantly delayed onset and decreased severity of EAE, even in mice dearing T cells bearing a transgenic T cell receptor (TCR) specific for myelin basic protein (MBP). As observed in CD4$^+$ T cells of humans, FAE did not only improve the disease: it prevented Th1-development and skewed the autoreactive myelin MBP-specific Th cells toward a Th2 phenotype *in vivo*. To determine whether this *in vivo*-induction of MBP-specific Th2 cells was related to the clinical improvement, we transferred TCR-transgenic, MBP-specific Th2 cells from FAE-treated mice. These MBP-specific T cells were unable to cause EAE following adoptive transfer into naïve mice, showing that FAE-treatment abolished pathogenecity of autoreactive T cells *in vivo*. Thus, abrogating the pathogenic potential of autoreactive T cells *in vivo* is promising as therapy of Th1-mediated autoimmune diseases such as psoriais, rheumatoid arthritis, multiple sclerosis or autoimmune diabetes.

114 [Oral 017]

Direct Detection and Quantification of Specific MHC-Peptide Complexes on Malignant and Nonmalignant Cells Using Recombinant TCR-Like Antibodies

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T cells recognise and respond to specific MHC-Peptide complexes expressed at the surfaces of other cells. Although a large number of T cell epitopes have been identified in recent years it was not possible to directly demonstrate and quantify specific MHC-peptide complexes at the surfaces of cells. All studies which relate to the quantity of such complexes are based on functional T cell assays and titrations of soluble peptides without knowledge of the resulting numbers of the specific MHC-peptide complexes. To overcome this limitation and to gain a deeper insight into the dose–response relationships of T cell responses we have developed from phage-display libraries recombinant antibodies with T cell receptor-like specificities, i.e. they recognise a specific peptide only in the context of the corresponding MHC allomorph. These antibodies display the same fine-specificity as the corresponding T cells, yet, in contrast to the notoriously low affinities of T cell receptors, they bind their ligands with the high affinity. Using these antibodies we analysed antigen presentation by dendritic cells (DC), melanoma cells, B lymphoblastoid cells, TAP-deficient T2 cells, monocytes, normal periphery blood CD19$^+$ B cells and CD4$^+$ lymphocytes. We could detect specific MHC-peptide complexes on all these cell types and establish the dose–response relationship of the peptide concentrations used for pulsing and the resulting densities of the cognate MHC-peptide complexes at the cell surfaces. These densities could be correlated with the responses of T cells with the same specificity. A comparison of these cell types for the loading rates and the turnover of specific complexes revealed that DC handle the peptides very differently than other cells. They require higher peptide concentrations for loading but, once pulsed, retain the peptides much longer, viz. the half-life of specific MHC-peptide complexes is much longer than for the other cell types. Some of the tumour cells, at the other extreme, incorporate peptides into their surface MHC-molecules at a relatively low peptide concentration already but also loose the specific complexes very quickly. These data demonstrate that peptide binding and the turnover of specific MHC-peptide complexes is different and more efficient on DC than on other cell types.

115 [Oral 018]

Application of Dendritic Cells Transfected with cDNA Encoding a T Cell Receptor Mimic Peptide, Prevents T Cell Activation in a Murine Contact Hypersensitivity Model

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A 8-amino acid peptide (TCRpep) encoding the sequence of the transmembrane region of the T cell receptor (TCR) α chain is able to block T cell activation by preventing the assembly of an functional TCR. Addition of this peptide to MLR reactions blocked T cell proliferation in vitro and after topical application onto psoriatic or eczematic skin, improvement of the skin diseases could be recorded. However, if the peptide was applied systemically a generalized immunsuppression occured. Our aim was therefore to develop a system that releases the mimic peptide locally at the site of T-cell activation. Therefore we cloned the DNS sequence encoding for the peptide into recombinant Adenoviruses and transduced DC. Initial results showed, that transduced DC-cellines released the peptide into the culture supernatant, resulting in reduced T cell proliferation in MLR assays. When transduced DC were injected into OVA-TCR transgenic DO11.1 mice, proliferation of OVA specific T cells was reduced as compared to controls, demonstrating suppressive activity of the TCRpep transduced DC in vivo. Next we tested the effect of TCRpep in CHS experiments. Mice were injected with TCRpep transduced DC that had been pulsed with the hapten TNBS, and respective controls. After 6 days mice were challenged with TNCB at the ears and ear swelling was determined 24h later. In these experiments ear swelling was markedly reduced in mice injected with TCRpep transduced DCs. The effect was largely antigen specific since sensitization against other contact allergens was not affected. Thus these data show, that DC expressing the TCRpep are able to prevent T cell activation in vivo and might be a useful tool to induce antigen specific immune suppression.

116 [Oral 046]

Engagement of ILT2/CD85j in Sézary Syndrome Cells Inhibits Their CD3/TCR Signaling

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Extensive phenotype analysis of cutaneous T cell lymphoma (CTCL) malignant cell lines revealed surface expression of receptors usually not detected on normal circulating CD4+ CD45RO+ lymphocytes. We previously found that CTCL malignant cells express KIR3DL2/CD158k, whereas they fail to express the other KIRs. We tested the surface expression of ILT2 with the GHI/75 mAb on freshly isolated PBL from Sézary syndrome patients and on Pno and Cou-L CTCL cells lines. In order to demonstrate that ILT-2 expressed by CTCL malignant cells can deliver a negative signal sufficient to inhibit cell proliferation, we studied the functional consequence of ILT2 molecule engagement on Pno CTCL cells triggered to proliferate through their CD3/TCR receptors. The cross-linking of ILT-2 receptors resulted in a significant decrease of the proliferation rate of CTCL cells triggered by immobilized anti-CD3 mAb. Interestingly, the cross-linking of ILT2 receptors alone did not influence the IL7-dependant proliferation of Pno malignant cells. We found that the malignant CD4+ ILT2+ population was less sensitive to anti-CD3 mAb induced cell death than the CD4+ normal population. In the present study, we report for the first time that ILT2/CD85j receptor is found on Sézary cell lines and on circulating Sézary malignant CD4+ cells while it is hardly detectable on circulating CD4+ lymphocytes from normal individual. We demonstrate that ILT2 is functional on CTCL cells as its triggering leads to the specific inhibition of CTCL malignant cell proliferation induced by CD3/TCR stimulation. Interestingly, we found that separated CD4+ ILT2+ circulating malignant Sézary cells are less susceptible to anti-CD3 mAb-induced cell death than autologous CD4+ ILT2- lymphocytes. Therefore, the resistance to apoptosis of Sézary cells may result from distinct mechanisms including specific expression of inhibitory receptors involved in lymphocyte survival.

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The Antimicrobial Peptide LL-37 is Required for Re-Epithelialization of Human Skin Wounds and is Lacking in Chronic Ulcers

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The human antimicrobial protein, hCAP18 is a component of the innate immune system and has broad antimicrobial activity conferred by its C-termus LL-37. hCAP18 is constitutively produced in leucocytes and is induced in barrier organs upon inflammation and infection. We demonstrate here a novel role for this peptide in keratinocyte migration and re-epithelialization of skin wounds. We have investigated the expression pattern of hCAP18 in acute and chronic wounds. We show that high levels of hCAP18 are produced in skin *in vivo* upon wounding and hCAP 18 is being in the inflammatory infiltrate and in the migrating epithelium. The highest hCAP18 levels are attained at 24 h postinjury, declining to preinjury levels upon wound closure. In chronic ulcers however, hCAP18 levels are low and bioactive LL-37 barely detecable. Using a noninflammatory *ex vivo* wound healing model, composed of organ-cultured human skin, we show that hCAP18 is strongly expressed in migrating skin epithelium, and that treatment with antibodies raised and affinity purified against LL-37, inhibits re-epithelialization in a dose-dependent manner. We suggest that, in addition to being an antimicrobial peptide, LL-37 also plays a role in wound closure and that its reduction in chronic wounds impairs re-epithelialization and may contribute to their failure to heal.

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Localized Th2-type Dermatitis and Strong Hev b 6.01, But Not Hev B 1, Specific IgE Response After Repeated Topical Natural Rubber Latex Sensitization

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Natural rubber latex (NRL) allergy may manifest as protein contact dermatitis on the hands of health-care workers and other NRL glove users, in addition to immediate type I allergy symptoms. The role of repeated epicutaneous (EC) sensitization with NRL proteins in the development of dermatitis and antibody response was studied in a murine model. EC sensitization with NRL produced significant influx of mononuclear cells, T cells and eosinophils to the sensitized skin sites. The number of degranulated mast cells in NRL-sensitized skin sites was significantly higher compared to PBS-treated sites ($p < 0.01$). IL-1β and IL-4 mRNA expression were markedly increased in NRL-sensitized skin sites. Moreover, significant increases in MCP1, Eotaxin-1, MIP1-α and MIP1-β mRNA were found in sensitized skin sites. EC sensitization with NRL induced a striking increase in total and specific IgE levels but not in IgG2a levels. In contrast, intraperitoneal (IP) immunization with NRL induced a strong NRL specific IgG2a response. Interestingly, EC-sensitization with NRL elicited strong IgE response against Hev b 6.01 but not against Hev b1. On the contrary, IP immunization with NRL elicited strong IgG2a production to Hev b1 but not to Hev b 6.01. These results demonstrate that EC-sensitization with NRL induces Th2-dominated dermal inflammation and strong IgE response in a murine model. EC-sensitization to NRL proteins eluting from the latex gloves may play an important role in the development of specific IgE and in clinical manifestations of acute and chronic hand dermatitis in patients.

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Expression Profiling of the Basal Cell Carcinomas After Imiquimod Treatment

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Imiquimod, a potent immune response modifier, exhibits its immunomodulatory activity primarily through interferon (IFN)-α induction and through release of other cytokines thus promoting the shift towards cell-mediated immune response. Basal cell carcinoma (BCC) of the skin has been shown to respond to imiquimod treatment. To investigate altered gene expression profiles of the basal cell carcinomas treated with imiquimod cream (Aldara®), we used Affymetrix GeneChip® arrays containing 12 000 known human genes. To investigate whether imiquimod exerts its action directly on the tumor cells or indirectly through the immune modulation, imiquimod gene profiles BCC cell lines treated with imiquimod or IFN-α *In vitro* were compared to the profiles of the skin BCC after imiquimod therapy *in vivo*. In the skin, imiquimod up-regulated the whole cascade of interferon-inducible genes (i.e. MxA and B protein, various OAS, STAT1 and 2) which matched the pattern and the intensity observed in BCC cell lines treated with IFN-α. In addition, imiquimod treatment *in vivo* induced a whole spectrum of genes involved in antigen processing and presentation (i.e. PA28, TAP-1, PSMB6 and 10), adhesion (i.e. SLAM, ICAM-2, PECAM-1), apoptosis (i.e. Fas, caspase 10, TRAF1, TRADD) and immune activation (CD40, CD86, LAG-3, RANTES, MIP-1R, CCR7R, etc.). On the contrary, no changes similar to the gene patterns could be detected in BCC cell lines after sole imiquimod treatment. These results indicate that IFN induction is mainly responsible for imiquimod immunomodulation and underlines the importance of immune cells in the skin are necessary for the responsiveness to imiquimod.

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FcεRIα is Expressed on Langerhans' Cells (LCs) in the Skin of Subjects with Both Active Atopic Dermatitis (AD) and Other Allergic Diseases

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Surface FcεRI is on LCs in the skin of subjects with atopic dermatitis (AD) and has been suggested to be a prerequisite for the condition. However, it is our hypothesis that surface expression of FcεRI on LCs is not restricted to AD but is present in all active systemic allergic diseases. To test this, epidermal sheets were obtained using suction blisters from 6 nonatopic controls (NAC), 11 with AD and 11 with a history of atopic disease other that AD (NAD). Sheets were either fixed immediately in acetone or left unfixed prior to incubation with antibodies against CD1a, FceRIa (antibody 22E7), IgE or vimentin (control) and examined by confocal microscopy to assess the frequency of $CD1a^+$ LCs immunoreactive for FcεRIa or IgE. Comparison of fixed and unfixed preparations allowed us to distinguish between cell surface and cytoplasmic immunoreactivity. LCs from all individuals contained cytoplasmic FcεRIα., Cell surface FcεRIα expression accompanied by IgE staining, was seen in 8/11 AD and 5/11 NAD. No surface FcεRIα was seen in NAC. Thus, epidermal LCs express surface FcεRIα and bind IgE, not only in AD but also in allergic asthma and rhinitis. That it is seen only in subjects with active or recently active allergic disease, suggests it occurs as a consequence of active systemic atopic allergic disease and is not restricted to AD.

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Withdrawn

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Drug Hypersensitivity Reactions – Role of T-Cells

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Hypersensitivity reactions to drugs can cause a great variety of diseases affecting mainly the skin but often also other organs. Drug specific T-cells play a decisive role both by orchestrating the immune response and inflammation, and by acting themselves as killer cells: They are found in the circulation as well as in the affected tissue of clinically distinct forms of drug allergy and their functional analysis *In vitro* is in agreement with the findings in immunohistology. Both, CD4+ and CD8+ T-cells recognize the drug by their $\alpha\beta$-TCR in an MHC dependent way. Drugs are stimulatory for T-cells, when they covalently bind to peptides or proteins, but also if the drug has structural features allowing it to bind in a rather labile manner (noncovalently) to the MHC-peptide complex. In maculopapular exanthema immunohistology of the affected skin and functional tests of drug specific T-cell clones demonstrate the presence of cytotoxic CD4+ and to a lesser degree of CD8+ T-cells in the dermis and epidermis, both containing perforin and granzymeB. These cytotoxic T-cells are in close contact to keratinocytes that show signs of cell destruction. In patients with bullous skin disease there is a higher percentage of cytotoxic, CD8+ T-cells emigrating into the epidermis. Drug specific T-cells also release various cytokines, in particular IL-5 and in pustular forms of drug allergy even IL-8. In conclusion, the findings demonstrate the decisive role of T-cells in various drug allergic diseases and allow to attribute distinct clinical forms of drug hypersensitivity to different T-cell reactions.

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Characterisation of Nickel Specific T-Cells in Patients with Allergic Contact Dermatitis

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Allergic contact dermatitis (ACD) is a delayed type hypersensitivity reaction mediated by T lymphocytes. The role of different allergen specific T-cell subsets and the way these subsets can migrate to the site of inflammation is still unclear. One of the major molecules on T-cells involved in homing to the skin is the cutaneous lymphocyte associated antigen (CLA), but also other molecules, like chemokine receptors, can play an important role in this migration. In this study we characterise nickel specific T-cell subsets and their chemokine receptor expression in nickel allergic patients. To test nickel specific reactivity in different T-cell subsets (CD4/CD8, CD45RO/CD45RA, CLA, CXCR3/CCR4/CCR10/CCR6), PBMC from nickel allergic patients were depleted from respective subsets and cultured in the presence or absence of nickel sulphate. After 6 days of culture proliferation was measured by ^{3}H-thymidine incorporation. To study chemokine receptor- and CLA-expression on different T-cell populations after stimulation with nickel sulphate triple FACS-stainings were performed. Depletion of CD4^{+} or CD45RO^{+} cells gave a decrease of nickel-specific proliferation compaired to nickel-specific reactivity measured in total PBMC. Depletion of CD8^{+} or CD45RA^{+} cells did not impair the nickel specific reactivity. Depletion of CLA^{+} cells completely abrogated nickel specific proliferation while purification resulted in an increased specific response. After 6 days culture of PBMC with nickel sulphate an increase of CD4^{+}/CLA^{+} cells was visible by FACS-staining. These cells express the chemokine receptors CXCR3, CCR4 and CCR10, but not CCR6. Indeed, preliminary data show that nickel specific proliferation is increased in T-cells which express the chemokine receptors CXCR3, CCR4 or CCR10. In conclusion, nickel reactive T-cells in peripheral blood of allergic patients are characterized by CD4-, CD45RO-, and CLA positivity. Besides CLA also CCR4, CCR10 and CXCR3, but not CCR6 seem to be relevant homing receptors expressed on allergen specific effector T-cells. Current studies demonstrate the contribution of this T-cell population in allergic inflammatory skin reactions and their interactions with regulatory T-cells.

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The Immunosuppressive Potency of Pimecrolimus is Greater Than That of Cyclosporin *In Vitro*

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Orally administered cyclosporin has often been used to treat very severe and recalcitrant cases of psoriasis and atopic dermatitis. However, as there are several known side-effects associated with cyclosporin, pimecrolimus (ASM-981) has been proposed as a better treatment option. In this study, the immunosuppressive potency of pimecrolimus was compared with that of cyclosporin in several different *In vitro* models. In the human mixed lymphocyte reaction, IC50 values of 0.26 ng per ml and 14.4 ng per ml were reported for pimecrolimus and cyclosporin, respectively. The second model measured the release of histamine from human basophils and the IC50 value obtained for pimecrolimus was 46.0 ng per ml and 333.5 ng per ml for cyclosporin. In addition, the Th1 cytokine (IFN-γ) production from human peripheral blood mononuclear cells (PBMC) stimulated with CD3/CD28 was evaluated, and pimecrolimus and cyclosporin had IC50 values of 9.94 ng per ml and 100.0 ng per ml, respectively. Finally, measurement of Th2 cytokine (IL-4) production from human PBMC stimulated with anti-CD3/CD28 revealed IC50 values of 3.61 ng per ml and 13.6 ng per ml for pimecrolimus and cyclosporin, respectively. In conclusion, these four different *In vitro* models have demonstrated unequivocally that pimecrolimus has substantially higher immunosuppressive potency than cyclosporin.

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T Cells: Link Between Streptococcal Angina and Psoriasis

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Psoriasis vulgaris is a disorder of abnormal keratinocyte proliferation induced by antigen-specific activation of T lymphocytes in the skin. Still unresolved is the question of how tonsillar infection with *Streptococcus pyogenes* (Strep) which is a major trigger of first psoriasis onset may contribute to lesional psoriatic T cell activation. To analyze this relationship at the T cell level we compared the T cell receptor (TCR) usage in skin lesions and tonsils of patients with Strep-induced psoriasis. The TCR β-chain V gene repertoire was amplified by PCR using 26 different BV gene specific primers together with a BC primer in skin lesions and tonsil of the patients. PCR products were analyzed by fragment length analysis, cloning and sequencing of TCR cDNA. By this approach clonally expanded TCR rearrangements were detected in skin lesions of both patients that indicated antigen-induced T cell activation. Strikingly, these particular TCR rearrangements were also identified within the tonsils. When tonsillar T cells were sorted according to the expression of the cutaneous lymphocyte-associated antigen (CLA), which acts as a skin homing receptor for T cells, the clonal TCR rearrangements from the skin lesions could be selectively assigned to the CLA-positive T cell fraction. Together these results demonstrate that T cells of the same T cell clone are present in psoriatic skin lesions and tonsillar tissue of psoriasis patients with recurrent streptococcal angina. They suggest that T cells activated during streptococcal tonsillitis are primed to enter the skin where they become reactivated to induce psoriatic inflammation. Our data therefore suggest that T cells may constitute the functional link between streptococcal throat infection and skin inflammation in the pathogenesis of psoriasis.

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Afferent and Efferent Phases of Allergic Contact Dermatitis (ACD) Can be Induced after a Single Skin Contact with Haptens. Evidence Using a Mouse Model of Primary ACD

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Allergic contact dermatitis (ACD) to haptens is a T cell-mediated DTH reaction, which occurs upon hapten challenge in already sensitized individuals. The inflammatory response in ACD requires a sensitization phase leading to induction of antigen-specific T cells in the lymph nodes (afferent phase) and an elicitation phase responsible for T cell activation in the tissue (efferent phase). Conversely, previously unsensitized individuals may develop a "primary allergic reaction" after the first skin contact with haptens leading to a skin inflammation with all the features of ACD. Here, we used an experimental model in Balb/c mice, refered to as contact hypersensitivity (CHS), to study the pathophysiology of primary allergic reactions and their relationship to classical ACD. We show that ACD can develop after a single exposure to DNFB and FITC applied onto the skin without subsequent challenge. Indeed, one epicutaneous application of a non irritant dose of hapten was sufficient to induce an optimal ACD reaction at the site of primary contact with the hapten, which started on day 6 after skin painting, peaked at day 7 and resolved by day 9–10. As in classical ACD, the skin inflammation was mediated by IFN-g producing, CD8+ effector T cells which were induced in the draining lymph nodes at day five post sensitisation and down-regulated by CD4+ T cells. RT-PCR analysis revealed that PACD reaction was mediated by an early recruitment of CD8+ T cells at the sensitization skin site at day 6 post sensitization. Analysis of the fate of the hapten in the skin revealed its persistance for up to 14 days after skin painting. Thus, primary ACD develops in the absence of secondary challenge, due to the persistance of the hapten in the skin which allows activation of specific CD8+ T cells at the site of the single hapten application.

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Blockade of Skin Dendritic Cell Migration by Topical Application of MMP- and PKC-Inhibitors

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Epidermal langerhans cells (LC) and skin dendritic cells (DCs) are potent antigen presenting cell (APC) in the induction of primary T cell-mediated immune responses in the skin. They capture foreign antigens (Ags) and migrate to regional lymph nodes (LNs) to carry and present this antigen to naive T cell. This mechanism is critical in the initiation of different cutaneous pathology such as Psoriasis, Contact Dermatitis or Contact Hypersensitivity (CHS). Several molecules regulate the migration of skin DC, among which metalloproteinase (MMPs) and proteine kinase C (PKC). The aim of this study was to evaluate the participation of MMPs and PKC in the *in vivo* migration of murine skin DCs, using topical inhibitors for MMP and PKC. Mice were ear painted with FITC and DC migration to auricular LNs was assessed 24 h later by flow cytometry quantification of FITC+, CD86+, MHC class II high positive cells. Topical application of MMP or PKC inhibitors on the skin once a day for 4 consecutive days induced a dose-dependant reduction in the number of FITC+ migrating DC (around 30% of inhibition for the optimal dose), compared to placebo application. When PKC and MMP inhibitors were mixed in the same ointment, the inhibitory effect was more pronounced (86% of inhibition), suggesting a synergistic action. These data indicate that, topical use of inhibitors of PKC plus MMPs, could inhibit skin DC migration, which could have strong therapeutic implications in the treatment of skin inflammatory dermatoses.

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Delayed-Type Reactions to Amoxicillin are Detected by Skin Patch Tests But Not by Lymphocyte Transformation/Activation Tests

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Adverse cutaneous drug reactions may involve activation of innate (pseudo-allergy) or specific immunity, the latter corresponding to hypersensitivity reactions. Drug-induced cutaneous DTH reactions often present as maculo-papular rashes and are due to the activation in the skin of drug-specific T cells. Diagnosis of DTH reactions to drugs rely on the demonstration of the presence of drug-specific T cells, using two main types of tests: (i) *in vivo* epicutaneous and intradermal tests which induces a localized contact dermatitis or DTH reaction in sensitized patients; (ii) *In vitro* lymphocyte transformation (LTT) and activation (LAT) tests which measure the proliferation of peripheral blood T cells, or the expression of cell surface activation markers, respectively. The aim of this work was to test for the sensitivity and the specificity of LTT and LAT as an *In vitro* diagnostic tool in 8 patients with typical DTH reaction to amoxicillin (i.e. amox-induced maculo-papular exanthema and positive skin patch tests/and ID tests to amox.). PBMC were recovered from patients 1–3 months after the resolution of the exanthema. Total PBMC, purified CD4+ and CD8+ T cells were cultured with/without amox. or an irrelevant drug in the presence of irradiated autologous antigen presenting cells and tested for amox-specific proliferation (LTT) and amox-induced expression of CD69/CD25 antigens (LTA). Results show that LTT and LTA are not usefull for the diagnosis of DTH to amox. Since: (i) LTT / total PBMC was positive in only 4/8 patients; (ii) LTT / CD4 and LTT / CD8 was postive in 2/5 patients; (iii) LTA / CD25 was positive in 2/5 patients. In conclusion, the high proportion of false negative LTT and LTA tests in selected patients with T-cell mediated amox-induced exanthema is incompatible with their use as in vitro diagnostic tests for DTH to amox.

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Inhibitors of Dipeptidyl Peptidase IV (DP IV, CD26) Suppress the Proliferation of Peripheral Blood Mononuclear Cells in Psoriasis

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The ectoenzyme dipeptidyl peptidase IV (DP IV, CD26) is known to play a crucial role in T cell activation. Recently it has been shown that synthetic inhibitors of DP IV enzymatic activity suppress DNA synthesis as well as cytokine production of stimulated T cells suggesting a potential therapeutic application of these agents in autoimmune diseases. As an example of a T cell mediated putative autoimmune disease we studied psoriasis, characterized by epidermal hyperproliferation and vascular inflammation of the skin. The expression of DP IV/CD26 on peripheral blood mononuclear cells (PBMC) was examined by immuno-flowcytometry in patients with psoriasis as well as the effects of the two DP IV inhibitors Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide on the proliferation of these cells *ex vivo*. The portion of $CD3^+/CD26^+$ PBMC did not differ significantly in psoriasis compared with healthy controls. *In vitro*, both DP IV inhibitors (20 μM) led to a substantial significant suppression of DNA synthesis of phyto-hemagglutinin (1 μg per ml)-stimulated PBMC from patients with psoriasis ($70.2 \pm 13.1\%$, $66.9 \pm 11.9\%$, respectively) and from healthy donors ($81.6 \pm 13.2\%$, $74.4 \pm 9.2\%$, respectively, compared to 100% of untreated controls). The viability of PBMC was not impaired by the two DP IV inhibitors under the chosen conditions. Thus, we could demonstrate that the inhibition of the enzymatic activity of DP IV in PBMC may be a possible therapeutic target in psoriasis.

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Effector Cells and Effector Mechanisms in $CD8^+$ and $CD4^+$ T Cell Mediated Murine Contact Hypersensitivity

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It has recently been demonstrated that the dominant effector T cells in contact hypersensitivity (CHS) induced by haptens are cytotoxic, IFN-γ producing $CD8^+$ Tc1 cells. For $CD4^+$ T cells a regulatory role has been shown. One of the crucial pathogenetic factors of this allergic disease seems to be T cell mediated cytotoxicity which may be responsible for the dominance of $CD8^+$ T cells as well as for the pathogenetic processes in the skin, e.g. cytolysis of keratinocytes. In the current study we analysed the potential effector role of $CD4^+$ T cells and the evaluation of their effector mechanisms in CHS as compared to $CD8^+$ T cell mediated CHS. We made use of C57BL/6 mice which in response to TNP and DNP develop a Tc1 mediated CHS and of $CD8^+$ T cell deficient β2m Knockout (KO) mice which have been reported to be unable to mount a CHS response to DNP. When we compared CHS responses to DNP in the two mouse strains we found a good CHS response in C57BL/6 mice but no CHS in β2m KO mice as reported. However, we could show that also in the β2m KO mice DNP-specific T cells were induced. Interestingly, when we used TNP we found that β2m KO mice mounted a good CHS response which was similar to the TNP response in C57BL/6 mice in its kinetic. These data show that depending on the hapten used a CHS response can be mediated by $CD4^+$ T cells in the absence of $CD8^+$ T cells. We are currently analysing the effector mechanisms responsible for $CD4^+$ T cell mediated CHS and the reasons for the absence of DNP-induced CHS in these mice despite the priming of DNP-specific T cells.

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Intradermal Application of Bone-Marrow Derived Dendritic Cells Primes for a Skin Homing $CD8^+$ T Cell Subset

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Skin associated immune responses are driven by a T Cell (TC) subset specialized for skin homing by expression of chemokine receptors like CCR4, CCR10 and adhesion molecules like E-selectin ligand. Little is known about the factors which determine the targeting of TC to specific tissues. For example, the site of priming or the specific antigen presenting cell (APC) could be crucial. Based on our observation that, in contrast to intradermal (i.d.) injection, intravenous (i.v.) injection of *In vitro* generated bone marrow-derived hapten-modified Dendritic Cells (DC) is inefficient in sensitizing mice for hapten-induced contact hypersensitivity we compared the homing properties of $CD8^+$ effector TC depending on the immunization route. We used the adoptive transfer of TCR transgenic $CD8^+$ P14 TC specific for peptide p33 from LCMV glycoprotein to generate a high number of antigen-specific and traceable $CD8^+$ effector TC in C57BL/6 recipient mice. After *in vivo* priming of the transferred TC with peptide-pulsed DC given i.v. or i.d., skin inflammation was induced by painting the contact sensitizer trinitrophenyl (TNP) on both ears and the TC emigrated *In vitro* from ear sheets were analysed by FACS. In general we found 2–10 times more $CD8^+$ P14 TC emigrating from inflamed ear skin if the DC had been given i.d. compared to i.v. injection. E-Selectin Ligand was significantly increased on blood P14 TC only after i.d. immunization suggesting an important role for this molecule in the observed homing differences. Obviously, during TC priming the APC and/or the lymphoid tissue microenvironment crucially influences the homing properties of $CD8^+$ TC.

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Analysis of the Repertoire and Function of Lesional Skin Infiltrating CD8 T Lymphocytes in HIV-Infected Patients With Psoriasis

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Incidence and severity of psoriasis seem to be exacerbated in HIV-infected patients as compared to the general population. The worsening of psoriasis along the evolution of the immune deficiency is contradictory with the usual pathogenic role of CD4+ T cells. We have previously shown that skin infiltrating lymphocytes in HIV-infected patients with psoriasis are mostly memory CD8+ T cells expressing the HLA-DR activation marker and cytotoxicity markers such as Ti-A1, perforin and granzyme B, suggesting that a chronic antigenic stimulation, possibly of retroviral origin, could participate in the lymphocytic activation. The efficacy of highly active antiretroviral therapy (HAART) on psoriatic lesions is another argument in favour of this hypothesis. We have studied the repertoire of psoriatic plaques' infiltrating lymphocytes by the immunoscope method in 9 HIV-infected patients and 10 noninfected patients. The results show an oligoclonal pattern of CDR3 region size profiles, in both populations. Moreover the skin repertoire bias is not site-specific as no difference is observed between profiles obtained from two dictinct plaques at the same time. No anti-HIV specificity could be evidenced from peripheral blood mononuclear cells nor psoriatic skin-derived cell lines using HIV peptide/HLA-class I tetramer and ELISPOT interferon secretion detection. These results argue against the hypothesis of a cutaneous CD8+ T cell selection by HIV antigens, and are more in favour of an indirect pathogenic role of the retroviral infection via chemokine/chemokine–receptor interactions.

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CD123$^+$/CD4$^+$ Cells – A Predominant Dendritic Cell (DC) Population in Allergic Contact Dermatitis

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While the importance of Langerhans cells (LCs) and dermal dendritic cells (DDCs) in the induction phase of allergic contact dermatitis (ACD) is well established, their role, if any, in the effector phase has yet to be demonstrated. In this study, we searched in ACD lesions for the presence of not only DCs normally residing in the skin, but also of other members of the DC family. Biopsies from positive 72 h epicutaneous patch tests (ECTs; n = 9) as well as from normal human skin (NHS) (n = 3) were subjected to immunohistologic analysis. CD1a was used as a marker for LCs, CD1c for DDCs and CD123/CD4 for plasmacytoid DCs (pDCs). NHS contained CD1a$^+$ and CD1c$^+$ cells in both the epidermal (16.3/mm- and 1.3/mm basement membrane, respectively) and dermal (10/mm^2 and 13.7/mm^2, respectively) compartment. In ECT lesions the number of dermal CD1a$^+$ cells (47.5/mm^2) was significantly increased at the expense of their epidermal counterpart (12.5/mm basement membrane). Similar phenotypic changes were observed in the CD1c$^+$ population of ECT specimens. As opposed to the situation in NHS, a substantial proportion (10.4–38%) of epidermal and dermal CD1a$^+$ and CD1c$^+$ cells expressed the activation marker CD83. These findings are indicative of events occurring in hapten-challenged skin, i.e. LC/DDC migration and maturation. We also detected a small number of the newly defined CD123$^+$/CD4$^+$ pDC population in the dermal compartment of NHS (2.9/mm^2). Interestingly, these cells represented the vast majority of the entire DC infiltrate in ECT lesions. They were numerically increased in the upper dermis (71.3/mm^2) and, to a lesser extent, in the epidermis (7/mm basement membrane) of ECTs. It is tempting to speculate that their attraction to ACD lesions is a regulatory mechanism aimed at dampening, and eventually, terminating undesired T-cell responses.

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Absent or Low Expression of T-Cell Receptor ζ-Chain in T-Cells Infiltrating Human Pathological Skin Conditions

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Antigen recognition by the T-cell receptor (TCR) is mediated by the clonotypic TCR αβ or TCR γδ chains that are derived from the products of site-specific DNA recombination events during development. The remaining chains of the TCR, which include the CD3γ, δ and ε chains and a ζ-family dimer, are responsible for signal transduction. The cytoplasmic domain of the ζ chain is involved in signal transduction necessary for T-cell activation and subsequent proliferation. Up to now, the *in situ* situation concerning the expression of TCR ζ-chain has not yet been investigated on infiltrating T-lymphocytes in neoplastic and inflammatory cutaneous diseases. In this study, we analysed the expression of TCR ζ-chain *in situ* in a large series of human pathological skin conditions using immunohistochemical methods. Air-dried acetone-fixed frozen sections were incubated with two anti-TCR-ζ-chain denominated TIA-2 and G3 and processed with a standard alkaline phosphatase antialkaline phosphatase (APAAP) technique. No or at most scarce expression of TCR ζ-chain was detectable in the inflammatory skin conditions investigated as compared to CD3 positive cells. In cutaneous T-cell lymphomas (CTCL), stricking reduction of TCR ζ-chain reactive cells were found in either reactive or malignant T-cell components. Cutaneous B-cell lymphomas (CBCL) were also negative. The mechanism(s) responsible for the absent or low expression of the ζ chain in skin infiltrating T lymphocytes are unclear and various explanations may be advanced. It is possible that this TCR defect is induced by the skin microenvironment as an effect of local immunoregulatory influences. Alternatively, lymphocytes located in the skin may generally not express this molecule and the low expression in T-cells infiltrating inflammatory and neoplastic skin conditions may be unrelated to the underlying pathological process. In conclusion, the salient finding of this report is the demonstration of low or absent TCR-ζ-chain expression in T-lymphocytes infiltrating neoplastic and inflammatory skin conditions. The mechanisms responsible for reduced TCR ζ chain expression in skin-infiltrating T-cells and the biological significance of these findings remain to be elucidated.

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T-Cell Receptor Excision Circle (TREC) Content is Reduced in CD8+ T-Cells From Patients with Atopic Dermatitis (AD)

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Earlier studies have shown increased telomerase activity and shortened telomere length in T-cells from blood of patients with AD indicating an increased cellular turnover of CD4+ and CD8+ T-cells. In order to investigate the cellular turnover and/or thymic output of T-cells in patients with AD, TREC content was measured in peripheral blood from 15 patients and 15 age matched healthy donors. Measurement of TREC has within the last years become an established method to estimate recent thymic immigrants. Peripheral blood mononuclear cells (PBMC) were isolated from the blood samples and subsequently CD4+ and CD8+ T-cells were isolated by positive immunoselection using Dyna-Beads. TREC levels were analysed by real-time quantitative PCR. To normalize for the input of DNA, the Cá constant region and β2microglobulin were amplified in every sample tested. The results show significantly lower TREC content in CD8+ T-cells from patients with AD compaired to TREC content in CD8+ T-cells from age matched healthy donors ($p = 0.02$), whereas no difference was seen between the two groups regarding the CD4+ T-cell subpopulation. Significantly lower ratio of TREC content in CD8+ T-cells over TREC content in CD4+ T-cells were also seen for patients with AD compaired to donors ($p < 0.01$). These findings surgest that patients with AD have decreased thymic output and/or an increased peripheral proliferation of their CD8+ T-cells. These new observations together with the previous findings demonstrate universal changes in the peripheral T lymphocyte system of AD patients. Further studies are needed to evaluate and confirm the findings.

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Expression and T Cell Costimulatory Function of the TNF Ligand BAFF

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BAFF (B cell activating factor from the TNF family) is a member of the tumor necrosis factor (TNF) ligand superfamily. BAFF provides an important survival signal to immature B cells, and recently a T cell costimulatory activity was reported. While BAFF function appears well understood, the cellular source of this TNF ligand has not been fully characterized. Here, we studied expression of BAFF in peripheral blood leukocytes. We found that BAFF is preferentially expressed in dendritic cells (DC). Indeed, *ex vivo* DC and DC derived from monocytes or CD34 stem cells express high level of BAFF mRNA. A low level of BAFF mRNA, up-regulated upon cellular activation, was also found in resting T cells. In DC, LPS stimulation up-regulated BAFF mRNA. Functionally, we show that blockade of this endogenous production of BAFF with a soluble form of one of its receptors (TACI-Ig or BAFF-R-Ig) significantly inhibited T cell activation. This inhibition was observed when total peripheral blood mononuclear cells and, to a lesser extent, when purified T cells were used. This demonstrates that endogenous production of BAFF by circulating antigen presenting cells (APC) and activated T cells provide some level of T cell costimulation. In conclusion, the expression pattern observed in this study reveals a predominant expression of BAFF in APC and therefore confirms its immunostimulatory function. The potential similarity with the other closely related TNF ligand APRIL (a proliferation inducing TNF ligand) will be discussed.

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Autologous Stimulation of T Cells from Psoriatic Plaques *In Vitro*

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The specificity of T cells in psoriasis is unknown. As a first step toward a characterization of T cell receptor specificities, we characterized stimulation of T cells isolated from active psoriatic plaques by autologous plaque cells using a γ-interferon ELISPOT assay. Spot formation in the presence of an activating CD3-antibody served as positive control. The number of IFN-γ producing T cells, quantified as spots per 5×10^4 cells, increased by at least 50% in the presence of autologous plaque cells in six out of nine patients. There was high interindividual variation with respect to absolute spot number, stimulation magnitutde relative to anti-CD3 stimulation, distribution of T cell subsets, and T cell activation status (as quantified by expression of CD69). Patients were classified as ''responders'' when the increase in spot formation elicited by autologous plaque cells reached at least 10% of that elicited by anti-CD3. When using this classification, there was a significant difference in relative abundance of double-negative (CD4- CD8-) T-cells ($44 \pm 33\%$ in responders vs. $10.1 \pm 8.4\%$ in nonresponders; $p < 0.1$; $n = 4$ per group). Other differences in T-cell subset distributions were nonsignificant. There was no obvious correlation between any T – cell subset frequency, activation status and number of γ-interferon secreting cells. When T cell clones were generated by limiting dilutaion, three of three proliferating clones could be re-stimulated by autologous plaque cells. Together, these data show that autologous stimulation of T-cells by cells derived from the same psoriatic plaque can be reconstituted *In vitro*. This approach should be valuable for the assessment of biological relevance of individual T cell clones.

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$\alpha_v\beta_3$-Integrin Differentiate Between Acute and Chronic Delayed Type Hypersensitivity Reactions

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Chronic and recurrent episodes of inflammation can lead to tissue destruction and regenerative processes while acute episodes may resolve without. Angiogenesis plays a major role in organ-specific autoimmune diseases caused by delayed type hypersensitivity reactions (DTHR) and contact hypersensitivity reactions (CHSR). Vascular cell integrin $\alpha_v\beta_3$ is selectively induced during angiogenesis and mediates cellular adhesion to extracellular matrix proteins. To better understand mechanisms of chronic inflammation, we investigated the role of $\alpha_v\beta_3$-integrin in acute- (A-CHSR – one episode of inflammation) and chronic contact hypersensitivity reactions (C-CHSR – recurrent episodes) using RGD-peptide which selectively binds to $\alpha_v\beta_3$-integrin. Mice were sensitized and challenged with TNCB to induce and elicit CHSR. 12 h after TNCB challenge animals were injected with [^{18}F]Galacto-RGD or [^{125}I]Gluco-RGD peptide and scanned *in vivo* with the small animal positron emission tomograph MADPET or uptake was determined by autoradiography. *In vivo* MADPET images showed intense RGD peptide uptake in C-CHSR but not in A-CHSR. No uptake was determined in C-CHSR mice pretreated with unlabeld RGD peptide. The uptake ratio -right ear (treated) vs. left ear (untreated)- was 2.9 for C-CHSR, no increase was seen in A-CHSR. RGD peptide uptake in C-CHSR increased with episodes of inflammation. Maximum activity appeared 12–24 h after challenge. Immunohistochemical staining confirmed β_3 expression exclusively on blood vessels in ears with C-CHSR but not with A-CHSR. H&E stained sections confirmed enhanced angiogenesis in C-CHSR. Since TNF$^{-/-}$ and IL-4$^{-/-}$ mice showed normal $\alpha_v\beta_3$-integrin expression, angiogenesis seems to be independent from these two cytokines. Thus, angiogenesis and $\alpha_v\beta_3$-integrin plays an essential role in C-CHSR but not in A-CHSR. Thus, we demonstrate a new model for examination of angiogenesis also potentially applicable in clinical dermatology. Furthermore, our data may help to bring new insights in the pathogenesis of T cell mediated autoimmune diseases. Since $\alpha_v\beta_3$-integrin antagonists are available and capable to block angiogenesis, these compounds may led to new strategies for the treatment of atopic dermatitis, psoriasis or rheumatoid arthritis.

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Toll-like Receptor 2 and 4-Dependent Regulation of Inflammatory Signaling in Human Sebocytes

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Human sebocytes are able to regulate inflammatory and immune processes, involved in the development of acne lesions, by intrinsic mechanisms. On the other hand, a bacterial role in the potentation of inflammatory acne has been widely accepted. Toll-like receptors (TLRs) are involved in innate immunity and are able to recognize microbial components. We address here a possible mechanism of gram-negative and gram-positive bacterial compo-nent action on human sebocytes through a modulation of TLR2 and TLR4 expression. We investigated the actions of the bacterial cell wall components lipopolysaccharides (LPS) and lipoteichoic acid (LTA) upon TLR2 and TLR4 mRNA expression in SZ95 sebocytes after prolonged (+24 h) exposure. Changes in TLR expression by these agents were compared to the action of retinoids and hydrocortisone. The mRNA and protein levels were detected by RT-PCR and immunocytochemistry. The regulation of TLR mRNA levels was investigated by using the TaqMan quantitative PCR method. To detect the possible pro- or anti-inflammatory effects of microbial components and immunosuppressive drugs we measured the IL-8 expression in SZ95 sebocytes and its secretion by TaqMan quantitative PCR and ELISA, respectively. SZ95 sebocytes expressed TLR2 and TLR4 on mRNA and protein levels. LPS enhanced TLR2 and IL-8 expression ($n = 4$, $p < 0.05$), while LTA suppressed TLR2 expression, but barely regulated IL-8 expression. Hydrocortisone did not regulate TLR2 expression. On the other hand, preliminary data suggest that LTA suppresses TLR4 expression, whereas LPS exhibited no effect. Hydrocortisone showed a suppressive effect on TLR4 and IL-8 expression ($n = 4$, $p < 0.05$). Neither IL-8 nor TLR expression was regulated by retinoids after prolonged (+24 h) exposure. The present work provides first evidence that bacterial cell wall components may directly affect human sebocytes via a specific receptor-dependent mechanism in the absence of inflammatory cells.

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α-Melanocyte Stimulating Hormone Suppresses Antigen-Driven Lymphocyte Proliferation In Vitro and is Unrelated to Melanocortin-1 Receptor Genotype

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The neuropeptide α-melanocyte stimulating hormone (α-MSH) has potent immunomodulatory and anti-inflammatory activity. The effects of α-MSH are mediated via a family of five melanocortin receptors (MC1–5R), and it has previously been reported that human monocytes express the melanocortin-1 receptor (MC1R). Activation of MC1R on monocytes by α-MSH alters costimulatory molecule expression and stimulates release of interleukin-10 (IL-10). Monocytes act as antigen-presenting cells and cause lymphocytes to proliferate to a variety of antigens *in vitro*. Genetic variants of *MC1R* have been shown to have compromised function, and are associated with red hair, fair skin and an increased susceptibility to skin cancer. However, it is not known whether *MC1R* variants alter monocyte function or their ability to stimulate lymphocytes. Here we show that α-MSH suppresses monocyte-mediated lymphocyte proliferation in response to the antigen Varidase *In vitro* (mean reduction at 10^{-13} M α-MSH = 30.49%, SEM 4.24; $p < 0.001$) with widespread variability between individual subjects. MC1R expression on human monocytes and monocyte-derived dentritic cells was confirmed by RT-PCR. Sequencing of *MC1R* suggested that the suppression by α-MSH is independent of *MC1R* genotype. Furthermore, preliminary experiments suggest that α-MSH induced suppression of lymphocyte proliferation occurs via an IL-10 independent mechanism.

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TLR2-Mediated Skin Inflammation by Mycoplasma Lipopeptide MALP-2 is Mast Cell-Independent

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Mast cells (MCs) have recently been found to express toll like receptor 2 (TLR2), the receptor of macrophage-stimulating lipopeptide-2 (MALP-2), which is released by mycoplasmas. Since we and others have shown that activated MCs protect from the pathology and mortality associated with infections by enterococci, we hypothesized that MCs can initiate innate immune responses to mycoplasmas after stimulation via MALP-2/TLR2. Here, we tested whether MCs contribute to the induction of MALP-2 mediated inflammation. The injection of MALP-2 into the ear skin of mice (240 U per ml, 20 μL) resulted in pronounced inflammatory reactions, measured by earswelling (after 1 h: 97.4 ± 10.6 μm, vs. 54.0 ± 8.1 μm, $p < 0.005$) similar to MC-dependent anaphylactic skin responses in course and strength. However, MALP-2 induced skin reactions in genetically MC-deficient Kit^{W}/Kit^{W-v} mice were virtually identical to those in normal $Kit^{+/+}$ mice. In addition, MALP-2 failed to degranulate MCs *ex vivo*, indicating that the induction of MALP-2-mediated skin inflammation is MC-independent. To clarify why TLR2+ MCs are unresponsive to MALP-2 we performed RT-PCR analyses for TLRs and MyD88. We found that MCs, unlike macrophages, do not show full length transcripts for TLR2 and that MCs express mRNA only for the extracellular domain of TLR2 while they lack expression of the TLR2 signalling domain. Our data explain why MCs cannot be activated via MALP-2/TLR2 and show that the induction of MALP-2/TLR2-mediated skin inflammation is not initiated by MCs. These findings should inspire further investigation of the unique structure of MC-TLR2 and its functions.

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The RANTES Containing Granule System in T Lymphocytes: First Description and Hyperreleasability of T Cells from Patients with Exacerbated Atopic Dermatitis (AD)

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Perforin (Perf)-granules represent an essential component of the lytic machinery in cytotoxic T lymphocytes (CTLs) and NK cells. Recently, a Perf-reduction and -hyperreleasability from atopic CTLs was demonstrated. Since RANTES was shown to be stored in Perf-granules, we asked, if these RANTES storage organelles in AD-T cells are altered as well. Ficoll-isolated peripheral blood mononuclear cells of 6 patients with exacerbated AD and of 6 healthy controls (HC), and the constitutive RANTES-expressing T lymphoma line Jurkat (Kab 14) as a control were fixed in 2% formaldehyde, permeabilized with 0.2% saponin, and stained with directly labeled monoclonal antibodies (mAB) against CD8, CD4, Perf (Hölzel, Cologne, Germany) and two different mABs against RANTES (Pharmingen, Heidelberg, Germany; R & D Systems, Abingdon, UK). Staining was controlled by isotype matched mAbs. In addition, degranulation was induced by PMA and ionomycin, and the reduction of intracellular staining (mean fluorescence intensity, MFI) over time was quantified in a FACScan. Intracellular RANTES was detected in all $Perf^{+}$ $CD8^{hi+}$ CTLs. The percentage of $Perf^{+}$ $CD8^{hi+}$ CTLs was significantly lower in AD-patients ($15 \pm 6\%$) as compared to HC ($45 \pm 16\%$, $p < 0.01$) and, thus, so was the percentage of $RANTES^{+}$ $Perf^{+}$ $CD8^{hi+}$ CTLs. RANTES staining was detected in other lymphocytes as well. In the total of lymphocytes, there was no significant difference between AD-cells and HC with regard to RANTES staining. However, by setting the MFI for RANTES at time point zero for 100%, a RANTES-hyperreleasability was found: in PMA/ionomycin-stimulated AD-lymphocytes, the MFI for RANTES was $56 \pm 19\%$, $45 \pm 12\%$, and $42 \pm 14\%$ (of the initial MFI) after 10, 20 and 30 min, respectively, as compared to HC (83 ± 18, 69 ± 20, and $60 \pm 16\%$, respectively; $p < 0.05$). Taken together we demonstrate (i) strong evidence for the existence of a so far undescribed intracellular pool of RANTES containing granules in $CD4^{+}$ and $CD8^{+}$ T cells (ii) that these organelles can be mobilized fast to the cell surface (iii) a hyperreleasability for RANTES-granules in AD-lymphocytes. This supports the etiopathogenetic concept of a cell type independent granule-hyperreleasability as a major pathogenetic factor in AD.

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Modulation of Protease-Activated Receptor-2 Expression in Skin Tissue and Cell Lines

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Protease-activated receptor-2 (PAR-2) is activated by cleavage of the N terminus of the receptor by a serine protease such as trypsin or tryptase. PAR-2 is up-regulated in inflammatory skin of patients with atopic dermatitis or psoriasis suggesting a role of PAR-2 in cutaneous inflammation. Moreover, vascular component participates to the inflammatory process through leucocyte infiltration Therefore, we were interested in assessing PAR-2 expression and its modulation in endothelial (EA.hy926) and keratinocyte (HaCaT) cell lines, human skin, hair follicles and keratinocytes freshly isolated from normal skin. PAR-2 activation was performed with specific stimuli (agonist SLIGKV and trypsin) and inflammatory stimuli (TNFa and LPS). Inhibition of PAR-2 activation was evaluated with soybean trypsin inhibitor (STI) and compared with a natural soybean extract. Expression of PAR-2 was detected by RT-PCR (mRNA) and immunohistochemistry (protein). Our results showed that PAR-2 mRNA was identified in EA.hy926 and HaCaT cell lines, and in keratinocytes and hair follicles originating from plastic surgery. Immunolabelling corroborated these results. PAR-2 like immunoreactivity was present throughout the skin with stronger labelling in the epidermis and skin appendages (sebaceous and sweat glands, and hair follicle). PAR-2 mRNA was up-regulated at 4 h and protein was increased at 24 h after treatments with the different specific and inflammatory stimuli. Finally, specific PAR-2 inhibitors (STI and soybean extract) tested on cell lines and skin isolated elements were able to reduce PAR-2 activation at transcriptional and traductional levels. In conclusion, we have shown that PAR-2 is over-expressed in the human skin and cell lines during inflammatory conditions. Moreover, serine protease inhibitors blocked PAR-2 expression suggesting that the inhibition of the PAR-2 pathway by soybean extract may be an effective way for reducing skin inflammatory responses.

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Inhibitory Effect of Thermal Spring Water* on Adhesion Molecules-Induced by TNF-Alpha on Human Endothelial Cell Line

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Tissue inflammation is characterized by local leukocyte infiltration. Vascular endothelium activated by cytokines like TNF-α expresses adhesion molecules which interact specifically with their leukocyte ligands. TNF-α induces cytokines expression *via* the activation of the transcription factor NF-κB. Thermal Spring Water (TSW) exhibits therapeutic properties for treating atopic dermatitis and psoriasis. In order to understand its beneficial effects in these inflammatory diseases, we studied the modulation of adhesion molecules expression by TSW on TNFα-stimulated endothelial cell line. Experiments were performed on the human endothelial cell line EA.hy926. Cells were preincubated for 24 h by TSW and stimulated for 4 or 24 h by TNFα (0.5–30 ng per ml). Adhesion molecules expression was assessed by RT-PCR (mRNA) and immunolabelling (protein). The role of NF-κB was studied by immunohistochemistry. By cell ELISA and immunohistochemistry, we showed an inhibitory effect by TSW on two adhesion molecules expression: E-selectin (clinical marker of atopic dermatitis) and ICAM-1. This inhibitory effect was shown with both waters from the spring and from the commercialized spray. E-selectin mRNA analysis allowed us to conclude in a transcriptional inhibition by TSW through the inhibition of the NF-κB transcription factor. On a model of endothelial cell stimulated by an inflammatory cytokine (TNFα) Thermal Spring Water exhibits a regulation of inflammatory parameters through the inhibition of adhesion molecule involving NF-κB expression. *Avène-les-bains France.

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Autoreactive T- and B-Cell Epitopes in Bullous Pemphigoid Preferentially Locate to the NH_2-Terminus of the Extracellular Domain of BP180 (BPAG2)

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Bullous pempghigoid (BP) represents a model of antibody (Ab)-mediated autoimmunity. Autoreactive Ab that target the hemidesmosomal adhesion molecule BP180 are critical for the loss of dermoepidermal adhesion. Current concepts ascribe autoreactive T cells an active role in immune pathogenesis by triggering auto-Ab formation. Our strategy was to characterize autoreactive T- and B-cell responses against BP180 utilizing recombinants of the extracellular domain (ECD) of BP180. In particular, we investigated the relationship between T-and B-cell reactivity based on our hypothesis that in individual BP patients, BP180-reactive T cells may target the same epitopes as subsequently produced auto-Ab. A total of 20 BP patients were studied, all of whom had IgG reactive with the ECD of BP180. In 19/20 patients, autoreactive T cells recognized epitopes within the NH_2-terminal region, while T cells from 11/20 patients reacted specifically with the COOH-terminus of BP180. In several patients (9/20), additional T cell epitopes were located throughout the ECD of BP180. Analyzing auto-Ab profiles with T cell reactivity, we found that 85% of patients with NH_2-terminal reactivity had also IgG directed against the NC16A-domain. Furthermore, we identified an immunodominant peptide within the NC16A-domain of the ECD of BP180 which was recognized by a $CD4^+$ T helper cell clone in a HLA-DRB1*1301-restricted manner. Our data suggest that T cell epitopes of BP180 may be similar or identical to the regions that are recognized by auto-Ab. The identification of immunodominant T cell peptides will hopefully facilitate immunomodulatory strategies in BP.

146 [Oral 019]

Conditional Inactivation of VCAM-1 Attenuates the Cutaneous Hypersensitivity Response: Evidence for a Role of VCAM-1 as a Costimulatory Ligand in T-Cell Activation

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The vascular adhesion molecule (VCAM)-1 is a membrane bound adhesion molecule binding predominantly to $\alpha4\beta1$ integrin and with lower affinity to $\alpha4\beta7$ integrin. Thereby this molecule takes part in adhesive interactions between leukocytes and endothelial cells. In the cutaneous hypersensitivity (CHS) response a contribution of this pathway to the migration of leukocytes into the inflamed tissue has been suggested. Therefore interferon-α induced Cre-lox P mediated deletion of the VCAM-1 gene after birth was used to investigate the role of VCAM-1 in the irritant and the CHS response. Mutant and wild-type mice exhibited equivalent responses to the irritant croton oil. When mice were sensitized to 2,4-dinitrochlorobenzene a significant reduction of magnitude and duration of the contact hypersensitivity response in comparison to wild type mice in the late phase of the reaction (48–96 h) was observed. This reduction was characterized by a reduced cellularity, specifically by a reduction of monocyte and granulocyte counts. Langerhans cell localization and emigration form the skin was unaffected. As VCAM-1 is also expressed on APC, antigen-specific proliferation was investigated using lymph node cells and CD11c+ dendritic cells for restimulation. Here, a significantly reduced proliferation of T cells in comparison to wild type controls was found, indicating that VCAM-1 acts as a costimulatory ligand. Further analysis revealed that in a mixed lymphocyte reaction allotype CD4+ T cells were only poorly activated by VCAM-1 deficient CD11c+ cells. These results indicate that VCAM-1 in addition to its role as an adhesive molecule for migration to inflammatory sites, also acts as an accessory molecule supplying a crucial costimulatory signal for T cell activation.

147 [Oral 020]

Induction of Psoriasis Involves TNFα Production by Heat Shock Protein Receptor CD91 Expressing Antigen Presenting Cells and is Independent on Recruitment of Lymphocytes

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Psoriasis is a chronic skin disorder characterized by epidermal hyperproliferation and infiltration of numerous activated T cells. Key questions related to the disease pathogenesis of psoriasis include (i) the necessity of *recruitment* of activated lymphocytes for disease induction (ii) factors for activation of resident sentinels of the innate immune system, i.e. antigen presenting cells (APCs). Uninvolved skin from psoriatic patients (PN skin) was transplanted on RAG knock out (KO) mice deficient in T and B cells, double KO mice deficient in RAG and type I interferon receptor (AR), double KO mice deficient in RAG and type II interferon receptor (GR), as well as triple KO mice (AGR). Only AGR mice demonstrated a conversion to psoriasis (PP skin) (29 out of 30 (= 97%) skin grafts). Converted PP skin demonstrated a typical psoriatic phenotype on the macroscopic and microscopic level including an increase of involucrin positive cell layers, keratin 16 expression throughout the epidermis, strong suprabasal expression of Ki67, expression of high levels of ICAM-1 and HLA-DR on lesional keratinocytes, increase of CD31 positive blood vessels as well as expansion of CD4 and CD8 positive T cells. Development of the psoriatic lesions was correlated with a strong increase in HLA-DR positive APCs. These cells expressed the heat shock protein receptor CD91 and were in close proximity with CD91 ligand (HSP70) expressing keratinocytes. Presence of nuclear NFkB as well as TNF α protein production indicated an activated state of CD91 positive APCs. Abrogation of TNFα signals in CD91 positive APCs using TNFα specific antibodies and TNF receptor fusion proteins led to a blockade of psoriasis disease development in 8 out of 9 (88%) mice. We conclude that development of psoriasis is independent on the recruitment of circulating lymphocytes and involves the production of TNFα by CD91 expressing APCs.

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Dysregulation of the Perforin (Perf) System in Patients with Alopecia Areata (AA)

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Hair follicle-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) can re-induce lesions in AA scalp biopsies grafted onto SCID mice. In the Dundee experimental bald rat, depletion of $CD8^+$ CTLs restores hair growth. To elucidate in humans the role of the CTL's perforin effector mechanism, ficoll-isolated peripheral mononuclear cells in 22 AA-patients (36 ± 13 years; no systemic therapy; > 20% scalp involvement in n = 9, = 20% scalp involvement in n = 13) were investigated by immuno flow cytometry and compared to 12 healthy control individuals (HC, 30 ± 8 years). In AA-patients, the percentage of $CD8^+$ lymphocytes was reduced significantly as compared to HC (AA: $29 \pm 7\%$; HC: $35 \pm 7\%$; $p < 0.05$). The number of $CD4^+$ cells was increased (AA: $49 \pm 9\%$; HC: $38 \pm 13\%$; $p < 0.05$). Whereas the percentage of $CD3^+$ T cells was not altered in AA, we found a significant reduction of $CD56^+$ cells as compared to HC (AA: $12 \pm 5\%$; HC: $26 \pm 13\%$; $p < 0.05$). Perf was expressed significantly less in AA-lymphocytes (AA: $25 \pm 9\%$; HC: $36 \pm 10\%$; $p < 0.01$). Among lymphocytic subpopulations Perf-reduction was significant in $CD8^+$ cells (AA: $52 \pm 18\%$; HC: $65 \pm 9\%$; $p < 0.05$), in $CD26^-$ cells (AA: $43 \pm 13\%$, HC: $60 \pm 9\%$, $p < 0.001$) and in $CD28^-$ cells (AA: $48 \pm 13\%$; HC: $67 \pm 9\%$; $p < 0.001$), respectively. However, neither "classical" CTLs expressing high amounts of CD8 nor CD56+ NK cells showed any reduction of Perf-positivity. Since AA is frequently associated to atopy and the Perf-system in atopic CTLs has formerly been shown to be impaired, we subgrouped AA-patients according to the total serum IgE level (AA: 223 ± 257 kU per l; HC: 14 ± 14 kU per l; $p < 0.005$). There was no significant difference in Perf-expression of lymphocytic subgroups between AA-patients with normal (IgE < 100 kU per l; n = 11; Perf: $24 \pm 8\%$) or elevated IgE levels (IgE = 100 kU per l; n = 11; Perf: $25 \pm 10\%$). Thus, one may conclude that (i) Perf-reduction in AA-lymphocytes may be not related to IgE levels, and (ii) is probably caused by a loss of Perf-expressing NK cells.

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Presence of the Arachidonic Acid Pro-Inflammatory Pathway in Human Sebocytes *In Vitro*

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Arachidonic acid (AA) is a polyunsaturated fatty acid which is incorporated in the phospholipids of the cell membrane. Under circumstances, phospholipases support AA release from the cell membrane. 5-Lipoxygenase (5-LOX) catalyzes the conversion of intracellular or extracellular AA to hydroperoxy-eicosatetraenoic acid (HPETE) and further to leukotriene A4 (LTA4). LTA4 is metabolised by LTA4 hydrolase to LTB4, which has been recognised as pro-inflammatory mediator. Leukotrienes cause degranulation, increased vascular permeability and endothelial adhesiveness, as well as neutrophil chemotaxis and activation. In a previous study, we have demonstrated that a systemic 5-LOX inhibitor was able to reduce the number of inflammatory lesions and sebum synthesis in patients with acne. Therefore, this work was conducted to investigate the presence and induction of AA metabolising enzymes and relevant AA inflammatory pathway receptors in human sebocytes. SZ-95 sebocytes were maintained with or without AA and calcium ionophore A23187. After 1, 6, 12, 24 h, the cells were washed twice with PBS and total protein and RNA were extracted for Western blotting or RT-PCR, respectively. 5-LOX was present under AA and calcium ionophore treatment at the mRNA and protein levels. In addition, preliminary immunocytochemical studies have localised 5-LOX in SZ95 sebocytes. Furthermore, LTA4 hydrolase and PPARa, being receptor for the natural ligand LTB4, were expressed in SZ95 sebocytes at the mRNA and protein levels. The presence of the enzymes 5-LOX, LTA4 hydrolase and the detection of PPARa in human sebocytes provide strong evidence that eicosanoids may play an important role in inflammatory sebaceous gland disorders, including acne.

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Long-lasting Ca^{2+} Influx in CD8+ T-Cells Induced by Mature Dendritic Cells Correlates with Their Proliferative Potential

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Dendritic cells (DC) as potent antigen presenting cells are able to induce MHC class I dependent CD8+ cytotoxic T-cell responses. Here, we wished to determine, whether the maturational state of the DC plays a role during the early cell-cell contacts leading to CD8+ T-cell proliferation. TCR-transgenic CD8+ T-cells were used from P14 mice and coincubated with autologous bone marrow derived DC in a time-lapse video microscopy system. We found that DC can interact with T-cells by both short–lived interactions or long–lasting interactions. Low-peptide concentrations or addition of immature DC mainly induced short-lived Ca^{2+}-influx of 3–5 min in CD8+ T-cells, even detectable in a low number of T-cells in the absence of peptide or during T-cell/T–cell self interactions. In contrast, the number of CD8+ T-cells with a persistent Ca^{2+}-influx for 30 min or more could be substantially raised by the use of higher peptide concentrations or matured DC. ^{3}H-Thymidine incorporation assays directly correlated with the number of T-cells showing persistent Ca^{2+}-signals, suggesting that those are critical events needed to induce T-cell proliferation. Interestingly, DC incubated only for 7 h with the maturational stimulus showed similar effectiveness to induce long-lasting Ca^{2+}-influx. Therefore, the induction of long-lasting Ca^{2+}-influx seems to be independent of the maturation-induced up-regulation of MHC and costimulatory molecules on the surface of DC, since these changes occur much later at the earliest after 18 h as determined by FACS-analysis. Instead, confocal-microscopy experiments using small RHO-GTPase inhibitors revealed that a cytoskeletal reorganization of MHC and costimulatory molecules on DC were responsible for sustained Ca^{2+}-influx resulting in CD8+ T-cell proliferation.

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Nickel Effects Exerted *In Vitro* on Lipid Peroxidation, ROS Production and Cytokine Secretion

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Nickel, a leading cause of allergic contact dermatitis, is thought to exert some of its adverse effects through promotion/exacerbation of oxidative processes. Arachidonic acid (AA) plays a central role in cell homeostasis, apoptosis and inflammation, and is a susceptible of oxidative modifications, even promoted by transition metals, leading to cytotoxic products. The effects exerted by Ni^{2+} on oxidation/decomposition of AA were investigated by TBARS assay, NMR analysis of hydroperoxides, and GC-MS measurement of 4-hydroxy-2-nonenal (4-HNE). Ni^{2+} resulted to poorly initiate AA autoxidation, whereas proved effective in 15-HPETE decomposition and 4-HNE formation. In Fe^{2+}-induced AA oxidation, Ni^{2+} inhibited AA decomposition. However, a decreased inhibition was observed raising Ni^{2+} concentration. When peroxides were present prior to Fe^{2+} addition, as in autoxidated or 15-HPETE-treated arachidonate solutions, Ni^{2+} accelerated Fe^{2+}-induced AA consumption and enhanced TBARS formation. The ability of Ni^{2+} of inducing ROS production was evaluated following stimulation of HaCat with Ni^{2+} (2–24 h) at different concentrations (7–35 μM). Moreover, IL-1β secretion was monitored after 48 h treatment with 35 μM $NiSO_4$. As result, ROS production and IL-1β secretion were significantly induced by Ni^{2+} incubation. The performed experiments showed that Ni^{2+} (i) is effective in enhancing AA decomposition and aldehyde formation in presence of peroxides (ii) triggers ROS generation, and (iii) induces IL-1β production. In conclusion, ROS over-production associated with the increment of inflammatory mediators may initiate peroxidative process that elevate the peroxide/Ni^{2+} ratio rendering Ni^{2+} more reactive. Activation of Ni^{2+} facilitates the formation of elevated levels of 4-HNE and other aldehydes from hydroperoxides breakage, concurring to sensitization process.

152 [Oral 016]

The Langerhans Cell-Specific Costimulatory Molecule, Dectin-2, is a Key Molecule in the Induction of Regulatory T Cells in Ultraviolet Light-Induced Tolerance

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Ultraviolet B light (UV) induces tolerance in a contact hypersensitivity (CHS) response in a hapten-specific manner. Although this phenomenon has long been appreciated, the mechanisms underlying this process are not completely clear yet. Currently, we could show that Dectin (Dec)-2, a novel costimulatory molecule selectively expressed on Langerhans cells (LC) plays a pivotal role in this process. To further delineate the immune pathway involved, we first isolated whole T cells from UV-tolerized mice that were previously exposed to low doses of UV for 4 successive days and sensitized thereafter with dinitrofluorobenzene through an irradiated skin area. Those T cells, called UV-tolerized T cells, upon transfer induced immune unresponsiveness in recipients when those were sensitized and challenged. By fractionating UV-tolerized T cells of either soluble (sol) Dec-2 bound or unbound population, it was found that only transfer of the sol-Dec2-bound T cell population resulted in suppression of CHS in recipients. Interestingly, however, the sol-Dec2-unbound T cells, when transferred to recipients, successfully led to vigorous CHS even when those mice were only challenged without sensitization, indicating that UV-tolerized T cells are consisted of two population, suppressor- and effector-T cells. Sol-Dec2-bound T cells were further fractionated with antibodies directed against CD4, CD8, CTLA-4 and CD25, resulting that only $CD4^+CD25^+$ T cells reproduced immune suppression in recipients upon transfer. Together, the present study indicates that sol-Dec2-bound $CD4^+CD25^+$ T cells, a population similar to the currently highlighted regulatory T cells, are responsible for UV-induced tolerance, which are the immune outcome determined by the balance between UV-induced T regulatory cells and effector T cells in a same UV-tolerized animal.

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Restoration of Energy Metabolism Protects From Induction of Photoaging-Associated Mitochondrial (mt) DNA Deletion

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Deletions of mitochondrial (mt) DNA are involved in ultraviolet (UV)-induced photoaging. MtDNA only encodes for genes of the respiratory chain responsible for generation of energy intermediates (ATP). It has been hypothesized that the most frequent mutation of mtDNA, the so called common deletion, induced by reactive oxygen species (ROS) leads to a decreased pool of energy equivalents. The cell is then believed to increase respiratory chain activity leading to the generation of more ROS. Evidence for the vicious cycle: ROS – mtDNA deletions – energy reduction – respiratory chain up-regulation – ROS – mtDNA deletions, has been elusive, however. In the present study it has therefore been assessed whether restoration of energy levels in cells undergoing chronic UVA exposure could prevent induction of the common deletion. While cellular ATP levels are volatile, phosphocreatine is a more stable energy equivalent in the cell. Therefore, employing a semiquantitative nested-PCR assay we investigated whether the generation of the induction of the common deletion by UVA and subsequent functional changes (oxygen consumption, MMP-1 induction) could be inhibited by coincubation of human dermal fibroblasts with the energy precursor creatine. Lipid peroxidation assays and absorbance spectrometry revealed that creatine has no antioxidative or UV absorbing capacities. However, coincubation of cells with creatine led to a dose dependent decrease of UVA-induced levels of the common deletion. Most interestingly, creatine coincubation not only reduced mtDNA mutagenesis but also normalized mitochondrial oxygen consumption and MMP-1 induction indicating a protective effect of creatine from mtDNA mutagenesis and subsequent functional changes. These results provide direct evidence for the existence of a vicious cycle in which mtDNA mutations lead to reduced energy levels, an increase of ROS and thereby to new mtDNA mutations. Furthermore, application of energy equivalents may represent a new and innovative way to protect the skin from UV-induced photoaging.

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Topical Retinoids Prevent UVB-Induced DNA Damage in Mouse Epidermis

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We previously showed that mouse epidermis can be highly loaded with retinyl esters by topical retinoids. Using a model we recently described to assess the filter properties of lipophilic molecules, we observed that retinyl palmitate (RP), the predominant endogenous retinyl ester, can act as a UV filter by preventing the photodestruction or the photooxidation of sensitive probes encapsulated into liposomes. Since the main cellular target of UV radiations is DNA, we wondered if topical retinoids could prevent the DNA damages induced by UVB. To test this hypothesis, hairless mice were treated once a day for three days with topical retinoic acid (RA), retinal (RAL), retinol (ROL), RP or vehicle, then exposed to a single UVB dose of 1 J per cm^2. Two kinds of DNA damages were assessed in the whole skin: apoptotic (sunburn) cells were visualised 24 hours later by the TUNEL technique, and cyclobutane thymine dimers were identified by immunohistochemistry dot blots and quantified by densitometry two or 24 hours after UVB exposure. All four topical retinoids were shown to decrease the spatial density of apoptotic cells by about 50%. Two hours following UVB exposure, the mean concentration of thymine dimers was significantly decreased only by topical RP. However, when assessed 24 hours after UVB exposure, the mean concentration of thymine dimers was decreased by 55% with topical RA or RP, by 75% with topical ROL, and by 90% with topical RAL, which is compatible with an increased rate of DNA repair. In summary, since RP afforded some protection against the formation of apoptotic cells and thymidine dimers, our data confirms *in vivo* the filter capacity of RP that we previously demonstrated *In vitro*. However, the biological reparation of thymine dimers, which requires several hours to start, was dramatically accelerated by topical RAL, and only moderately by topical RP. The mechanism by which this effect is achieved is under study.

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Retinyl Esters Exert UVB-Blocking Effects Similar to Those of Commercial Sunscreens

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Our recent studies aiming at assessing the protection afforded by topical retinoids against the deleterious effects of UVB on mouse epidermis suggested that retinyl esters could have UV-filter properties. In order to explore this hypothesis, we developed a model to evaluate UV-filter capacity of lipophilic molecules. Thus liposomes are used as a model of cellular membranes. The molecules to be tested for UV-filter properties are included into these membranes, whereas a fluorescent probe, Indo-1 or 2′,7′-dichlorofluorescin (DCFH) is encapsulated inside the liposomes. In the present study, we applied this model to compare the UV-filter capacity of retinyl palmitate (RP) with that of three well-known sunscreens, i.e. Ocytlmethoxycinnamate (OMC), Avobenzone (AVO), and Benzophenone-3 (BP3). A UVB dose of 180 mJ per cm^2 destroyed 67% of Indo-1 in unprotected liposomes, whereas it destroyed only 37%, 18%, 18% or 7% of Indo-1 in liposomes containing RP, OMC, BP3 or AVO, respectively. The photooxidation of DCFH induced by 180 mJ per cm^2 UVB in unprotected liposomes was completely abolished in liposomes containing RP or OMC, whereas BP3 and AVO did not provide any protection against the photooxidation of DCFH. This indicates that, although AVO and BP3 have good UVB-absorbing properties, they were not able to prevent the photooxidation of DCFH induced by UVB. RP and OMC are both good UVB-filters and inhibitors of photooxidation. In summary, there are two kinds of sunscreens: those with only filter capacity, and those which can also prevent the photooxidation of hydrophilic molecules, such as those found inside living cells. Interestingly, it appears that RP, the predominant endogenous retinyl ester, belongs to the latter category.

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EUK-134, a Catalase-Mimic, Inhibits the Activation of Stress-Induced MAPK Pathways and Reduces UVB-Induced p53 Accumulation in Primary Human Keratinocytes

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UVB damages keratinocytes through both oxidative stress and direct DNA damage. EUK-134, a synthetic superoxide dismutase (SOD) and catalase mimetic, exhibits powerful antioxidant activity against lipid peroxidation and might therefore confer protection against UVB-irradiation in keratinocytes. We investigated the effect of EUK-134 on the UVB induced accumulation of the p53 protein and on UVB induced activation of the MAPK JNK and p38 by Western blot analysis. The protein p53, a universal sensor of cellular damage, accumulates in the skin after exposure to UVB, mediating growth arrest after a low UVB dose and apoptosis after a high UVB dose. Cells treated with 5–50 μM EUK-134 before UVB-irradiation showed a significantly lower induction of the p53 protein in a dose-dependent fashion. The stress activated kinases JNK and p38, members of the Mitogen activated protein kinase (MAPK) family, have been shown to be directly involved in the accumulation of p53 and the induction of apoptosis. Both are also strongly activated upon UVB damage. Pre-treatment of keratinocytes with 50 μM EUK-134 prior to UVB-irradiation was found to strongly reduce the activation of p38 MAPK and JNK as well as the activation of their upstream kinases, respectively, MKK3-MKK6 and SEK1/MKK4. It has been shown that ligand-independent clustering of membrane receptors can trigger stress induced MAPK pathways after UVB-irradiation. UVB-induced membrane damage through oxidative stress is thought to play an important role in this process. We hypothesize that EUK-134, by direct protection of the membrane from UVB-induced oxidative damage, reduces stress induced MAPK signaling and as a result lowers the level of p53 induction. This could, in turn, lead to an enhanced survival of human keratinocytes after UVB-irradiation.

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High Dose Long Wave Visible Light Induces Perinuclear Vacuolization *In Vivo*

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With the advancing widespread use of photodynamic therapy (PDT), questions arise about the necessity to protect the adjacent healthy skin from high dose long-wave visible light used in PDT. The aim of the present study was to investigate the effects of high dose visible light on the skin of healthy volunteers with the focus on apoptosis, DNA damage, inflammation, melanogenesis and induction of matrix metalloproteinases (MMP). Fourteen healthy volunteers were included and irradiated daily on their buttocks with 130 J per cm^2 long-wave visible light (560–780 nm) on 5 consecutive days with a cumulative dose of 650 J per cm^2. In each volunteer in total 6 biopsies were taken before and 24 h after irradiation on days 1, 2, 4, 7 and 11. Frozen and paraffin sections were investigated by measuring parameters for photodamage (apoptosis, p53, phosphorylated c-Jun), skin ageing (phosphorylated c-Jun, MMP-1, elastin content) and melanogenesis (melan-A). Although no sunburn cells were seen, a significant increase in perinuclear vacuolization was noted ($p < 0.0003$) from day 5 till 7 days after last irradiation. There was no expression of phosphorylated c-Jun, whereas the expression of p53, Melan-A, MMP-1 and elastin content did not change. In our experiment high dose visible light induced a significant increase in perinuclear vacuolization, but did not result in apoptosis, photodamage or early induction of skin aging.

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Further Characterization of the Activity of UV-Induced T Suppressor Cells

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Ultraviolet radiation (UV) induced immune tolerance is due to the generation of hapten-specific T suppressor cells which recently have been renamed regulatory T cells. We recently reported that these T cells express the surface marker CTLA-4 which in contrast to its cognate member CD28 acts as a negative regulatory molecule. CTLA-4 seems to be functionally relevant since blocking of CTLA-4 with a neutralizing antibody is associated with a loss of suppressive activity. Triggering of CTLA-4 induces the release of a cytokine pattern which is similar to that of type 1 regulatory T cells. To further characterize the mechanism by which CTLA-4+ T cells cause suppression, lymph node cells were obtained from mice which were tolerized against dinitrofluorobenzene (DNFB) by UV. Cells were enriched for CTLA-4+ cells and cocultured with bone marrow-derived dendritic cells (DC) in the presence or absence of the water soluble analogue of DNFB, dinitrobenzene-sulfonic sodium salt (DNBS). After 48 h apoptosis of DC was analyzed by Annexin V staining. While CTLA-4 negative cells did not affect DC in their survival rate, pronounced apoptosis of DC was observed upon coincubation with CTLA-4+ cells. However, CTLA-4+ cells exerted their apoptotic activity only in the presence of DNBS. To analyze the pathway by which CTLA-4+ cells induce apoptosis, expression of the death receptor CD95/Fas on DC was checked by FACS analysis. CD95, however, was only slightly induced by CTLA-4+ T cells. Therefore, the expression of the antiapoptotic proteins Flip and Bcl-xL was determined in DC by intracellular FACS analysis. Both proteins were constitutively expressed, but significantly down-regulated in the presence of CTLA-4+ T cells and DNBS. Taken together, these data indicate that UV-induced T suppressor cells seem to exert their inhibitory capacity on antigen presentation via induction of apoptotsis of DC and that down-regulation of the antiapoptotic proteins Flip and Bcl-xL is involved in this process.

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The Influence of Heat Shock on DNA-Repair after UVB

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It has been reported that heat pretreatment protects cultured human keratinocytes and normal murine human skin from ultraviolet (UV) induced cell death. Whether this is a beneficial (less cell death) or a harmful (survival of mutated cells) effect is not yet clear. In the present study we investigated whether heat shock interferes with the formation and repair of UV-B-induced photoproducts (thymine (TT)-dimers). This DNA damage appears shortly after UV-exposure and is consequently repaired by efficient repair mechanisms. In our experiments we compared the formation of TT-dimers after UV-B exposure in heat treated and control normal human keratinocytes, melanocytes, and fibroblasts employing a standard ELISA protocol using a monoclonal antibody against TT-dimers. To investigate the repair kinetics the amount of photolesions was determined at several time points after UV-B treatment (30 min, 1, 3, 6, and 24 h). For all three cell types investigated we found a slightly increased repair rate in heat shocked cells within the first three hours after UV-B exposure compared to controls. With continued culture the curves flattened out and 24 h after UV-B exposure the amount of dimers was identical in heat pretreated cells and controls. We conclude that heat shock does not negatively interfere with the removal of UV-B-induced TT-dimers. In contrast, our results might indicate that heat shock enhances DNA repair immediately after UV-B exposure. Whether heat shock protects from UV-induced mutagenesis remains to be investigated.

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The Cytotoxicity of UVA Depends on the Mode of Exposure

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The reciprocity rule (Bunsen-Roscoe law) states that a photochemical reaction is directly proportional to the total energy dose, irrespective of the dose distribution. The dose is the product of intensity and duration of exposure. In photomedicine the validity of this rule is generally taken for granted, although the influence of radiation intensity and dose fractionation are largely unknown. We have examined the effects of fractionation at a constant total dose on viability, apoptosis, and proliferation in the human squamous carcinoma cell line A431. For these experiments UV-A and UV-B from a metal halide source were delivered either as a single dose or as three equal fractions separated by intervals of 10, 30, 60, 120, and 240 min 12 h after initiation of exposure cells were investigated for Annexin V binding, viability (MTT assay), and BrdU incorporation. With UV-A fractionation with intervals of 10, 30, and 60 min led to a decrease in viability of up to 40%, compared to an identical single dose. In contrast, intervals of 120 and 240 min increased the viability up to 30%. Corresponding results were obtained for Annexin V binding and BrdU incorporation. With UV-B we observed no influence of dose fractionation on the rate of cell death. These results indicate that in a simple experimental model the Bunsen-Roscoe law is not valid with regard to biologically relevant endpoints. Our observations might be explained by cumulative oxidative stress caused by fractionated UV-A radiation with short intervals. On the other hand, the protective effects of repetitive low irradiation with long intervals may be attributed to an up-regulation of antioxidative defense mechanisms.

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Human Sun-Exposed Skin and Sun-Protected Skin Differ in Their Number and Composition of Cutaneous T Cells

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The purpose of this study was to compare the number and phenotype of cutaneous T cell populations in habitually sun-exposed sites vs. sun-protected sites. Several punch biopsies were obtained from 24 healthy volunteers. In order to study the epidermal and dermal CD3$^+$, CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ T cell populations we performed immunohistochemistry on cryostat sections. Compared with skin from sun-protected site, both intraepidermal and dermal CD3$^+$ T cell numbers of habitually sun-exposed site were significantly lower ($p < 0,05$). Double staining showed that the number of intraepidermal CD3$^+$/CD8$^+$ T cells was significantly lower ($p < 0,05$) in sun-exposed than sun-protected skin, whereas the numbers of CD3$^+$/CD4$^+$ T cell were similar in both sites. Therefore the CD4/CD8 ratio in the epidermal compartment was markedly higher in the sun-exposed area compared to the sun-protected area. The numbers of both CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ dermal T cells were lower in sun-exposed than sun-protected skin; however, the CD3$^+$/CD8$^+$ T cell population was more markedly reduced. The dermal CD4/CD8 ratio in the sun-exposed and sun–protected skin was 2.37 and 1.42, respectively. Our study showed that compared with the sun-protected skin, the sun-exposed skin had reduced number of T cells and altered CD4/CD8 ratio. We suggest that alterations in number and composition of intraepidermal and dermal T-cells in habitually sun-exposed skin may be responsible for local immunosuppression and therefore increased risk in skin cancer.

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Apoptosis of Keratinocytes After UVA Irradiation in the Presence of the Photosentitizer Tiaprofenic Acid Involves Reactive Oxygen Species and p53 Protein Stabilization

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Tiaprofenic acid (TPA) is a nonsteroidal anti-inflammatory drug exhibiting photosensitizing properties if simultaneously administered with UVA exposition. Previously, we demonstrated the formation of photoadducts in human epidermal cells and the induction of cell death via an apoptotic process after UVA irradiation in the presence of TPA. In the present work, we have studied the mechanisms involved in the induction of the programmed cell death of human keratinocytes following incubation with TPA and UVA irradiation. Our results demonstrated that TPA led to an early (< 2 h) and a delayed apoptosis (24 h to 48 h after irradiation). The immediate apoptosis was observed for TPA concentrations = 50 μM associated with UVA doses = 5 J per cm^2, whereas a delayed apoptosis was depicted for lower UVA and TPA doses. Apoptosis was characterized by phosphatidyl serine exposure (annexin V-Fitc × propidium iodide staining), DNA fragmentation and PARP cleavage. Reactive oxygen species, especially hydrogen peroxide and superoxide anion, as well as a strong lipid peroxidation were produced during cell irradiation in the presence of the drug. They might play a major role during the early apoptotic process since antioxidants like N-acetylcysteine and vitamin E partially prevented these immediate phototoxic effects. The delayed apoptosis involved the stabilization of the protein p53, cytochrome c release from mitochondria and caspase 9 activation. The cell cycle regulator protein p21 was not induced during these events. The use of p53–/– keratinocytes confirmed p53 implication in the delayed apoptosis. P53 induction and PARP cleavage were transiently induced for the lower doses (TPA 25 μM + UVA 5 J per cm^2) and cells showed a cell cycle arrest in the G2/M phase. Forty-eight h after irradiation, cell proliferation restarted. During both the early and delayed events, mitochondrial membrane potential ($\Delta\Psi$) decreased and was partially reversed by antioxidants. Taken together, these data indicate that TPA phototoxicity induces cellular alterations through rapid p53 induction, that leads to an apoptotic process with mitochondria and caspase 9 activation when no cell reparation was possible.

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The Phenotypical and Functional Analysis of Soluble Dectin-2-Bound Regulatory T Cells in UV Induced Tolerance

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We currently showed that the induction of tolerance is mediated by Dectin-2 (Dec-2), a novel costimulatory molecule selectively expressed on dendritic cells. Because T suppressor cells (Ts) are believed to play a pivotal role in ultraviolet radiation (UV)-induced tolerance, we were interested whether Dec-2-mediated signals are required for the immune regulatory properties of Ts. C3H/HeN mice, which were exposed to low doses of UV and sensitized with dinitrofluorobenzene (DNFB) through the UV-irradiated skin areas, exhibited profound suppression of contact hypersensitivity (CHS). When soluble Dec-2 (sol-Dec2), a competitive inhibitor of Dec-2, was injected into the UV-tolerized mice before resensitization, mice responded with a pronounced specific ear swelling response upon rechallenge, indicating that sol-Dec2 breaks UV-induced tolerance. Transfer of T cells obtained from UV-tolerized mice into naive animals inhibited the induction of CHS in the recipients. Suppression, however, was not observed when T cells were depleted of the fraction binding to sol-Dec2. Three channel FACS analysis revealed that sol-Dec2-bound T cells are $CD4^+CD8^-CD25^+$, thus being phenotypically similar to the recently identified regulatory T cells (Tr). Co-incubation of hapten-pulsed epidermal cells with T cells that were isolated from UV-tolerized mice resulted in enhanced expression of the death receptor Fas on Ia^+ Langerhans cells (LC) and in augmented apoptosis rate. Apoptosis of LC was significantly reduced by sol-Dec2. Together, these data indicate that UV-tolerized Ts belong to the family of Tr cells and are able to induce apoptosis of LC mediated via Dec-2. This indicates that Dec-2 may be a critical molecule, which induces death signals in LC and thereby causes UV-induced tolerance.

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Neutral Sphingomyelinase is Modulated by Glutathione in Keratinocytes UVB-Induced Apoptosis

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The sphingomyelin pathway is an ubiquitous, evolutionary conserved signaling system which plays a role in intracellular signal transduction. The action of a ligand binding to a surface receptor results in early activation of the enzyme sphingomyelinase (SMases) with consequent hydrolisis of membrane sphingomyelin (SM) and generation of ceramide. Increase in intracellular ceramide level is followed by three major cellular responses: cell growth arrest, induction of cell differentation and/or induction of apoptosis. Recent evidences have shown that reduced glutathione (GSH), but not other antioxidative agents, is able to inhibit apoptotic cell death and necrosis induced by hypoxia in PC12 cells. This protective effect is mediated by GSH direct inhibition of Neutral SMase activity and ceramide formation. In the present study we report that GSH inhibits activation of Neutral SMases and generation of ceramide and partially prevents apoptotic cell death induced by UVB radiation in keratinocytes. Normal human keratinocytes were cultivated with mitomycin-treated 3T3 cells in Dulbecco's modified Eagle's medium/Ham's F12 medium. At preconfluency cells were incubated with GSH for 2 h and then irradiated with a UVB dose of 75 mJ per cm^2. At different times after UVB irradiation, cells were harvested for *In vitro* measurement of neutral SMase activity, lipid extraction and Western blot analysis. *In vitro* measurement of Neutral SMases activity showed an early induction 15 min after UVB exposure with a subsequent decrease to control level after 2 h. Exposure to UVB radiation resulted in a rapid sphingomyelin hydrolysis and generation of ceramide as measured by TLC analysis. The ceramide accumulation started at 15 min after UV exposure and progressively increased up to 24 h. Addition of GSH significantly inhibited activation of Neutral SMase and generation of ceramide in UVB-treated keratinocytes. Moreover UVB-induced cleavage of PARP, a marker of the apoptotic response, was partially inhibited by GSH treatment. These data indicate that GSH plays a critical role in UVB signaling pathway regulating Neutral SMase activity.

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The Effect of Constitutive Pigmentation on Erythema Response in UVB Dose Dependent. T. Ha

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In a series of experiments, we have studied the relationship between objective skin colour (measured using a reflectance instrument and chromameter) and UVB-induced erythema. Experiment 1: A range of broadband UVB doses (119–300 mJ per cm^2) using a Diffey prototype lamp (TL12) was delivered to the lower back of 25 subjects. Constitutive skin colour and UVB-induced skin erythema was assessed 24 h after irradiation using the melanin index and erythema index derived from reflectance spectroscopy (Diastron, UK). Results: Using a linear ANOVA model (S and S-plus version 2, Insightful 2001), the relationship between melanin index and skin erythema was statistically significant for all doses delivered. The gradient coefficients were –0.30 (119 mJ per cm^2), –0.47 (150 mJ per cm^2), –0.60 (189 mJ per cm^2), –0.71 (238 mJ per cm^2) and –0.76 (300 mJ per cm^2), all $p<0.01$. The degree that melanin index explained erythema were $R^2=0.27$ (119 mJ per cm^2), $R^2=0.29$ (150 mJ per cm^2), $R^2=0.49$ (189 mJ per cm^2), $R^2=0.50$ (238 mJ per cm^2) and $R^2=0.61$ (300 mJ per cm^2); all $p<0.01$. Experiment 2: In 50 subjects with a wide variation of skin colour and skin phototypes, we delivered a range of broadband UVB doses (119–300 mJ per cm^2) to the lower back. Skin erythema was assessed at 48 hours after irradiation using the erythema index derived from reflectance spectroscopy (Diffey). In addition, α-characteristic angle as an index of baseline skin colour was derived from chromameter readings taken from lower back skin. Results: Using a linear ANOVA model, the relationship between α-characteristic angle and skin erythema was statistically significant for all doses delivered. The gradient coefficients were 0.85 (119 mJ per cm^2), 1.50 (150 mJ per cm^2), 2.34 (189 mJ per cm^2), 2.90 (238 mJ per cm^2) and 2.95 (300 mJ per cm^2); all $p<0.01$. The degree that α-characteristic angle explained erythema were $R^2=0.10$ (119 mJ per cm^2), $R^2=0.13$ (150 mJ per cm^2), $R^2=0.21$ (189 mJ per cm^2), $R^2=0.31$ (238 mJ per cm^2) and $R^2=0.35$ (300 mJ per cm^2); all $p<0.01$. Conclusion. We have demonstrated that the degree to which melanin index and α-characteristic angle accounts for the cutaneous erythema response to UVB increases as the dose of UVB increases. These results affirm the importance of constitutive skin colour on UVB-induced erythema, and demonstrate that the effect of constitutive skin colour becomes "stronger" as UVB dosage increases.

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Expression and Regulation of Cytoprotective Genes by Ultraviolet Radiation in Skin of Patients with Psoriasis

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We have investigated whether interindividual differences in the cutaneous expression of cytoprotective genes may contribute to variability in sensitivity and response to controlled UV exposure (UVR) in the treatment of psoriasis. We have used real-time quantitative RT-PCR analysis to characterise the expression of genes including cytochrome P450s (P450s), glutathione S-transferases (GSTs), drug transporters and stress response genes in 29 patients with psoriasis commencing phototherapy. Skin biopsies were taken from buttock sites: (a) 24 h after irradiation with a solar simulator (1–4 × MED site) (b) untreated psoriatic plaque and (c) control site. UVR treatment significantly induced the expression of the stress response gene cyclooxygenase 2 (median 4.6-fold induction, range 0.14–22.6) and lead to more modest (~2-fold) inductions of P450 CYP2S1, glutathione peroxidase, GSTP1 and the drug transporter MRP1. In contrast, P450 CYP2S1 (3.24-fold, 0.6–15.5), GSTP1 (3.36-fold, 1.3–33.1) and MRP1 (3.53-fold, 1.3–24.8) were significantly increased in psoriatic plaque, as were P450 CYP2E1 (3.45-fold, 1–28.9) and heme oxygenase (8.43-fold, 2.9–49.7), implying a global up-regulation of drug and oxidant metabolism in lesional psoriatic skin. We found considerable interindividual variation in constitutive cytoprotective gene expression and inducibility, indicating that individual patient phenotype may be a significant factor in determining response to UVR exposure and topical drug treatment in psoriasis patients.

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Beneficial Effect of Poly(ADP-Ribose) Polymerase (PARP) Inhibitor in Acute Photodamage

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The ultraviolet (UV) components of sunlight induce damage to the DNA in skin cells, which is considered to be the initiating step in the harmful biological effects of UV radiation. Repair of DNA damage results in the formation of single-strand DNA breaks, which activate the nuclear enzyme PAR. Overactivation of PARP worsens the oxidative cell damage and impairs the energy metabolism, raising the possibility that moderation of PARP activation following DNA damage may protect skin cells from UV radiation. The topical effects of the novel PARP inhibitor O-(3-pyperidino-2-hydroxy-1-propyl) pyridine-3-carboxylic acid amidoxime monohydrochloride (BGP-15 M) were investigated on UV-induced skin damage in a hairless mouse model. For evaluation of the UV-induced acute photodamage to the skin and the potential protective effect of BGP-15 M, DNA injury was detected by measuring the formation of single-strand DNA breaks and counting the resulting sunburn (apoptotic) cells. The ADP-ribosylation of PARP was assessed by Western blot analysis and then quantified. In addition, the UV-induced immuno-suppression was investigated by the immunostaining of tumor necrosis factor α (TNFα) and interleukin-10 (IL-10) expressions in epidermal cells. The signs of inflammation were examined clinically and histochemically. Besides its primary effect in decreasing the activity of nuclear PARP, topically applied BGP-15 M proved to be protective against solar and artificial UV radiation-induced acute skin damage. The DNA injury was decreased ($p<0.01$). An inhibition of immunosuppression was observed by down-regulation of the epidermal production of cytokines IL-10 and TNFα. In the mouse skin, clinical or histological signs of UV-induced inflammation could not be observed. These data suggest that BGP-15 M directly interferes with UV-induced cellular processes and modifies the activity of PARP. The effects provided by topical application of the new PARP-regulator BGP-15 M indicate that it may be a novel type of agent in photoprotection of the skin.

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Withdrawn

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Chronic UV Exposure Leads to Alterations in DNA Repair Capacity of Skin Cells

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UVB induce DNA lesions which are implicated in skin photocarcinogenesis. DNA repair capacity influences individual susceptibility to skin cancer development as illustrated in DNA repair deficient XP (Xeroderma Pigmentosum) patients who develop multiple skin cancer on sun exposed skin. In this work, our objective was to study if chronic sun exposure could lead to acquired alterations in DNA repair capacity in human skin as observed in the hairless animal model. Seven volunteers of phototype II to III undergoing surgical excision of a skin carcinoma located on sun exposed skin and having no germinal DNA repair deficiency, were studied. Keratinocytes and/or fibroblasts were isolated from 6 mm punch biopsy of chronically sun exposed and sun protected normal skin from each individual after informed consent. DNA repair capacity was studied using the comet assay up to 120 min post UVB irradiation (300 J per m^2). In all subject studied and whatever the cell type analyzed, chronically sun exposed skin derived-cells showed a significant reduction in the DNA incision process, maximum 1 h post-UVB irradiation. This deficiency was not explained by mutation in p53 gene as verified by direct sequencing of genomic DNA. Differential gene expression was analyzed using commercially available DNA macroarrays (Clontech) and allowed us to identify 2 genes down regulated in chronically sun-exposed skin potentially implicated in DNA repair capacity. These results were further confirmed by Northern blot. This work show that genetic alterations due to chronic sun exposure and independent of p53 can play a role in the susceptibility of chronically sun exposed skin to cancer development. These results could be of importance to further optimize skin photoprotectors.

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Differential Gene Expression Profiling in Individuals with Deficient and Proficient DNA Repair as a Predictive Tool for Individual Skin Cancer Risk

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Xeroderma pigmentosum (XP) is a disorder characterized by defective repair of ultraviolet (UV)-induced DNA damage comprising 7 complementation groups (A to G). Patients with XP have a highly increased skin cancer risk with the clinical phenotype varying according to the specific compl. group. The highest skin cancer risk is found in group A, an intermediate risk in group D and the lowest risk in group F. The high skin cancer risk is partly due to defective DNA repair, but other pathways also play a role (immune surveillance). Available test systems assessing the risk to develop skin cancer thus far only detected gross differences between single parameters. In order to increase the sensitivity and predictive power of a test system, employing DNA-array technology, we compared differential mRNA expression in fibroblasts from XP patients of the above complementation groups with a high, intermediate and low skin cancer risk after UVB irradiation. A dose dependent tendency from mild to severe was detected in 17 genes with 11 genes involved in functionally relevant pathways (DNA repair, cell-cycle, apoptosis, transcription, matrix-degradation). Thus it is possible to use DNA array technology to identify distinct patterns of UV-induced gene expression correlating with the skin cancer risk in XP patients. We were therefore next interested to see if this approach could also be used to determine the skin cancer risk in DNA repair proficient individuals which nevertheless exhibited an increased skin cancer risk. The gene patterns previously identified in XP patients could also be observed in patients with >2 skin tumours and an age <35 years. Expression levels of genes were lower than in XP cells but higher than in age matched normal cells. Thus it is possible to correlate the skin cancer risk with the complementation groups in XP patients, thereby identifying a limited number of genes with possible functional relevance for photocarcinogenesis. Furthermore this data could be transferred into the DNA repair proficient background and may provide a predictive test system to determine the skin cancer risk of clinically normal individuals.

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eNOS Knock Out Mice Undergo Increased Dermal and Epidermal Apoptosis Following UVB Irradiation

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Nitric oxide (NO) is released from the skin by constitutive and inducible forms of NO synthase following ultraviolet (UV) irradiation. NO has both pro and antiapoptotic actions, and we wished to determine which of these effects predominated in the skin. We compared the responses to UVB of mice null for endothelial NOS or iNOS ($eNOS^{-/-}$ and $iNOS^{-/-}$ mice, respectively). Apoptosis in frozen skin sections was detected with a fluorescently labelled antibody to the active site of Caspase 3. In the epidermis, wild type (wt) mice underwent $0.36 \pm 0.12\%$, $0.71 \pm 0.23\%$, and $3.79 \pm 0.82\%$ apoptosis, 24 h after irradiation with 0, 400 and 1000 mJ per cm^2 UVB. $eNOS^{-/-}$ mice underwent $0.1 \pm 0.02\%$, $2.86 \pm 0.84\%$ and $3.7 \pm 0.74\%$ apoptosis; and $iNOS^{-/-}$ $0.22 \pm 0.1\%$, $1.49 \pm 0.21\%$ and $3.19 \pm 1.02\%$ apoptosis. In the dermis, the figures were: wt, $0.94 \pm 0.42\%$, $4.74 \pm 0.79\%$, and $11.94 \pm 2.71\%$; $eNOS^{-/-}$, $2.58 \pm 1.13\%$, $13.23 \pm 1.21\%$, and 28.8 ± 1.14; $iNOS^{-/-}$, $0.98 \pm 0.2\%$, $8.81\% \pm 2.21\%$, and $14.09 \pm 3.71\%$. $eNOS^{-/-}$ mice show significantly more apoptosis than wt and $iNOS^{-/-}$ at 400mJ per cm^2 in the epidermis and at all UV doses in the dermis. Irradiation with UVB or addition of the NO synthase (NOS) inhibitor L-NAME increased apoptosis in the human keratinocyte cell line CCD 1106 KERTr, and apoptosis was greater when the two agents were given in combination. Addition of the chemical NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) immediately after UVB completely abrogated the rise in apoptosis induced by L-NAME, and largely reduced apoptosis due to UVB. Caspase-3 activity, an indicator of apoptosis, doubled in keratinocytes incubated with L-NAME as compared with the inactive enantiomer, D-NAME. This rise in caspase-3 activity was partially reduced by SNAP. Apoptosis was reduced by the addition of 8-Bromo cyclic GMP, and increased upon addition of 1,H-[1,2,4]oxadiazole[4,3-a]quinoxalon-1-one (ODQ), an inhibitor of soluble guanylate cyclase. We conclude that NO released by eNOS following UVB irradiation, limits apoptosis in the epidermis both by direct inactivation of caspases, and indirectly via cyclic GMP dependent pathways. At higher doses of UVB, the protective effects of NO are overwhelmed in the epidermis. Autologously released NO limits UVB induced death in the skin, and we believe has a physiologically vital function in ensuring the relatively high UV resistance of keratinocytes.

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UVA1 Therapy of Cutaneous Chronic GVHD

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Despite improvements in post-transplant immunosuppression, up to 60% of patients who receive an allogeneic bone-marrow transplantation will develop chronic graft vs. host disease (cGVHD) that contributes substantially to morbidity and mortality. Skin is a very frequent target of cGVHD. In recent years, the role of UVA1 (340–400 nm) radiation in the modulation of skin immune functions and collagen metabolism has with been assessed. We have treated 8 patients: 4 with generalized lichenoid (2) or sclerodermoid (2) and 4 with localized sclerodermoid cGVHD skin lesions. Patients had only mild or no other organ involvment. Skin lesions in areas that were inaccessible by UVA1 radiation were chosen to serve as unirradiated controls. All patients had failed to respond to conventional therapies and 7 out of 8 patients had developed significant drug toxicity, opportunistic infections, or both. Fixed daily exposures of 50 J per cm^2 of UVA1 radiation were delivered 3 times weekly. UVA1 therapy gave a complete clinical remission in 5 patients and a partial improvement in two after $19{,}25 \pm 6.90$ treatments. The remission was accompanied by improvement of histopathological findings. Unirradiated control lesions were not modified by treatment. Immunocytological studies did not show changes of circulating lymphocyte subsets. Adverse effects to UVA1 therapy were not registered. The gradual improvement of skin lesions allowed the timely reduction and discontinuation of most oral immunosuppressive agents leading to the improvement or, at the very least, cessation of progression of drug adverse effects and opportunistic infections. Extracutaneous cGVHD manifestations remained unchanged. At follow-up, patients with lichenoid cGVHD showed early relapses and entered in a prolonged maintenance regimen whereas sclerodermoid lesions showed more persistent remission. In conclusion, UVA1 radiation seems to represent an effective and well tolerated treatment option for cutaneous cGVHD without relevant short-term adverse effects although the risk of long-term toxicity, namely skin carcinogenesis, remains to be established.

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UVB-Induced Cell Cycle Arrest and DNA Repair Capacity in Human Epidermis is Enhanced After Vitamin C and Vitamin E Administration

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UVB irradiation causes a time dependent decrease of the proliferative activity of the human epidermis, which results from p53 tumor suppressor gene induction. The ensuing accumulation of p21 leads to cell cycle arrest in the G1-phase. This checkpoint mechanism is necessary to eliminate UV-damaged DNA sequences. Reentry into the cell cycle has been shown to require transcriptional activation of the c-Jun protooncogene, resulting in down-regulation of p21 levels. C-Jun expression in regulated by the MAPK pathway, signalling casade which can be activated by free radicals generated by UV. 17 volunteers (patients plus controls) were given a oral combination of 2 g ascorbic acid and 1000 IU d-α-tocopherol daily for 3 months. Biopsies were taken from unirradiated and UVB-irradiated skin (minimal erythema dose 2) before and after 90 days of vitamin administration. Biopsies were immediately incubated with 60 mM BrdU for 2 h. BrdU-positive cells were visualized in sections of paraffin-embedded tissue using a-BrdU antibodies and APAAP. BrdU is incorporated during the S-phase of cycling cells. The number of cells staining positive for BrdU is a marker of epidermal activity. After irradiation, a decrease in the number of proliferating cells (median of proliferating cells/length of sample: 9/mm before irradiation, 6/mm after irradiation) could be observed. Proliferative activition decreased even further after administration of vitamins (median of proliferating cells/length of sample: 8/mm before irradiation, 4/mm after irradiation; $p = 0{,}021$, *T*-test). Our finding suggest that administration of antioxidants results in an extension of the UV-induced cell cycle block and thereby allows for more time to repair DNA-damage, ultimately reducing the risk of mutations.

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***In Vivo* UVB Irradiation Induces Clustering of Fas (CD95) on Human Epidermal Cells**

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Both animal and *In vitro* studies with human cell lines have demonstrated a role for Fas in UV induced apoptosis. The purpose of the present study was to investigate the regulation and function of Fas and FasL in human skin after single dose UVB irradiation. Normal healthy control persons were irradiated with 3 MED UVB on forearm or buttock skin. Suction blisters from unirradiated and irradiated skin were raised and Fas, FasL and apoptosis were demonstrated on epidermal cells by flow cytometry. Clustering of Fas was demonstrated by confocal laser scanning microscopy on cryostat sections. Soluble FasL was quantitated by ELISA. Flow cytometric analysis demonstrated increased expression of Fas with changes in mean florescence intensity from unirradiated control: 290, 24 h: 304, 48 h: 580 and 72 h: 802 ($n = 4$). Apoptosis was demonstrated by TUNEL reaction and maximum of apoptotic epidermal cells was observed 48 h after irradiation. Double staining with Fas and TUNEL showed no correlation between Fas expression and formation of apoptotic cells. Expression of FasL transiently decreased with mean florescence intensity in unirradiated skin: 1900, 24 h: 1605, 48 h: 1382 and returned to the preirradiation level 72 h after irradiation: 1925 ($n = 4$). Soluble FasL concentrations in suction blister fluid from UVB irradiated skin did not differ from unirradiated skin ($n = 5$). Confokal laser scanning microscopy showed rapid clustering of Fas within 30 min after irradiation. Our results are in accordance with previous findings and suggest that Fas is activated *in vivo* in UVB exposed human skin.

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Induction of Apoptosis and Cell Death in Peripheral Blood Mononuclear Cells (PBMC) by Different Wavebands of Ultraviolet A (UVA) Radiation

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UVA1 radiation (340–400 nm) is increasingly employed in the phototherapy of T-cell mediated skin diseases. Several lines of evidence suggest that UVA1-induced T-cell apoptosis is a key factor for the therapeutic effect. In the present study we addressed the question whether this effect is specific for UVA1 radiation or can also be induced by biologically equivalent doses of UVA (315–400 nm) or UVA2 (315–340 nm) radiation. For the experiments a high pressure UV metal halide lamp was used whose spectral power emission was modified by different filter combinations to generate UVA, UVA1 or UVA2 radiation. Irradiance and relative spectral intensity were determined with a Bentham DM 150 double monochromator. Dose–response curves for cytotoxicity as determined by MTT assay were constructed for each of the three UVA spectra by exposing isolated PBMC of healthy volunteers to increasing doses of the respective wave band. The cells were then treated with equitoxic doses (LD_{10}, LD_{25} and LD_{50}, based on the MTT assay) of UVA, UVA1 and UVA2 and quantitative analysis of apoptotic and dead cells was performed by flow cytometry and double staining of cells with annexin V and propidium iodide. Sham irradiated cells served as a control. Independent of exposure dose and UVA spectrum the percentage of annexin V-positive/propidium iodide-negative (A+/PI–) cells was not significantly raised above control levels at 24 h after exposure. In contrast, there was a marked dose and UVA spectrum-dependent increase in annexin V-positive/propidium iodide-positive (A+/PI+) cells with UVA1 inducing much higher levels of A+/PI+ cells at all applied doses than UVA or UVA2. Our data indicate that UVA1 differs in its effects on PBMC from UVA and UVA2 and is substantially more effective in inducing cell death.

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Withdrawn

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All-*Trans*-Retinoic-Acid Up-Regulates Caspases-3, –6, –7, and –9 and Sensitizes Primary Keratinocytes to UV-Induced Apoptosis

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Previous experiments in our laboratory have shown that all-*trans*-retinoic-acid (ATRA) up-regulates caspase-3 at the protein-level in keratinocytes (KC). Western blot analysis revealed that ATRA-treatment also up-regulated the effector-caspases-6, –7, as well as the mitochondrial initiator caspase-9. Despite this up-regulation ATRA treatment alone was not sufficient for inducing apoptosis in KC. However, when in addition cells were irradiated with UVB – at doses not lethal for nontreated KC- the majority (>70%) of ATRA-treated cells detached and showed signs of apoptotic cell-death, which was confirmed by the demonstration of caspase-3 activation and DNA-laddering. This increased susceptibility to apoptosis could only be seen when confluent KC were treated for at least 2 days with ATRA. In contrast, in nonconfluent KC, or KC entering terminal differentiation the response of DMSO and ATRA-treated KC to UVB was comparable. UVB-induced apoptosis of ATRA-treated KC could be completely blocked when the p53-inhibitor α-pifithrin was added to the KC after UVB-irradiation. Blockade of death-receptor activation by incubating the cells on ice prior to and during irradiation, did not have any protective effect. These results show that KC are sensitized to UV-induced, p53-dependent apoptosis by ATRA treatment, probably due to an up-regulation of pro-apoptotic caspases. The increased sensitivity of KC to UV-induced apoptosis might be involved in the synergistic effect of combined UV and retinoid treatment in psoriasis.

178 [Oral 001]

FOXM1 is a Downstream Target of Hedgehog Signalling in Basal Cell Carcinomas (BCC)

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Forkhead box (FOX) proteins have been shown to play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, longevity and transformation. The functional importance of this gene family in normal human skin physiology and disease processes is not well understood as there is a paucity of information on the expression of these genes in the human skin. Activation of Sonic Hedgehog (Shh) signalling plays a key role in the development of BCCs of the skin in humans. Recent studies have established that some FOX genes are downstream targets of Shh signalling. We have investigated the role of FOX proteins in transducing Shh effects in human skin by using degenerate PCR to identify FOX genes differentially expressed in BCCs. All three known FOXM1 isoforms (a, b and c) were detected in human skin and cultured keratinocytes and the transcriptionally active FOXM1b isoform was found to be up-regulated in BCCs. Real-time quantitative RT-PCR showed that the increase in FOXM1 mRNA level was specific for BCCs and not a reflection of increased cell proliferation in that no up-regulation was seen in squamous cell carcinomas (SCCs) or proliferating primary human keratinocyte cultures. The dissociation between the cell cycle and FOXM1 expression was further supported by immunostaining studies which showed intense nuclear and cytoplasmic staining throughout BCC tumor islands and not confined to the periphery regions of the tumor where proliferating Ki-67-immunopositive cells are predominantly localized. In contrast to the mixed cytoplasmic and nuclear localization seen in BCCs, constitutive expression of an EGFP-FOXM1b fusion construct in cultured primary and immortalized epithelial and mescenchymal cells showed nuclear localization. Expression of the Shh target glioma transcription factor-1 (Gli1) in primary keratinocytes and other cell lines caused a significant elevation of FOXM1 mRNA level and transcriptional activity indicating that FOXM1 is a downstream target of Shh signaling. Although Gli1 expression in C3H10T1/2 cells stimulated the endogenous differentiation marker alkaline phosphatase (AP) activity, this was not seen with ectopic FOXM1b expression which suggests that FOXM1 is not mediating the differentiation signal in this model of Shh signaling. Our findings provide the first evidence that activation of Shh signaling is an important determinant of FOXM1 expression in mammalian cells. Given the role of FOXM1 in cell proliferation, the up-regulation of FOXM1 in BCCs may be one of the mechanisms whereby Shh signalling exerts its mitogenic effect on basal keratinocytes leading to the development of this common human cancer.

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High Killing Efficacy of a New Silicon Phthalocyanine in Human Melanoma Cells Treated with Photodynamic Therapy by Early Activation of Mitochondrion-Mediated Apoptosis

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Photodynamic therapy (PDT) is a promising therapeutic modality that utilizes a combination of a photosensitizer and visible light for the destruction of diseased tissues. Using human pigmented melanoma cells, we examined the photokilling efficacy of new silicon-phthalocyanines (SiPc) that bore bulky axial substituents. The bis(cholesteryloxy) derivate (Chol-O-SiPc) displayed the best *In vitro* photokilling efficacy ($LD_{50} = 6 \times 10^{-9}$ M) and was 9-times more potent than chloro-aluminium Pc (ClAlPc), a known photosensitizer used as a reference. The rate of Chol-O-SiPc uptake by cultured melanoma cells was higher than that of ClAlPc, leading remarkably to 30-fold more maximum accumulation. Although Chol-O-SiPc was half as potent as ClAlPc for promoting photo-oxidative membrane damage in a cell-free assay, early events of mitochondrion-mediated apoptosis upon PDT were triggered much faster. This was demonstrated by kinetics studies examining cells with permeabilized mitochondrial membranes detected by a specfic probe, cytochrome c release followed by confocal microscopy and caspase-9 activation monitored by an enzymatic assay. Inhibition of caspase-9 activity by a substrate analogue showed its central role in the pro-apoptotic events leading to photokill by Chol-O-SiPc PDT, while cytochrome c release was not affected. Thus, although the other Pc used as reference acted on the same apoptotic pathway, Chol-O-SiPc efficacy relies mainly on a high cell uptake and an early triggering of pro-apoptotic events mediated by the mitochondrion, enlightening thus a major role of this organelle in Pc PDT. Conclusively, Chol-O-SiPc represents a potential high efficiency photosensitizer, especially suitable for the photokill treatment of melanized cells and tumors, which usually prove refractory to PDT.

180 [Oral 028]

Effect of Disruption of Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase (SERCA) Function on the Distribution of Desmosomal Proteins

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SERCA is a membrane bound Ca^{2+} activated ATPase. It actively transports Ca^{2+} from the cytosol into the endoplasmic reticulum (ER) lumen to maintain cytosolic Ca^{2+} concentration. Increase in intracellular Ca^{2+} is shown to initiate the assembly of desmosomes in the epidermal cells *In vitro*. In addition, mutations in the SERCA2 gene cause Darier's disease, which is characterised by disruption of desmosomal complexes and abnormal epidermal differentiation. Using indirect immunofluorescence and Western blotting, we investigated the effects of SERCA disruption on cell-to-cell adhesion and expression of the desmosomal proteins desmoplakin, desmoglein, and desmocollin in normal human keratinocytes. SERCA function was inhibited by preincubation of cells with thapsigargin, a highly specific SERCA inhibitor. When SERCA function was blocked with thapsigargin, trafficking of the desmosomal proteins to the cell surface was inhibited. These proteins showed substantial colocalisation with the ER marker protein calnexin, suggesting that they were retained in the ER. We then extracted protein from these cells with mild detergent and separated into detergent soluble and insoluble fractions. In the presence of thapsigargin, the majority of desmosomal proteins were observed as detergent insoluble aggregates. Our findings suggest that inhibition of SERCA function prevents efficient differentiation and transport of desmosomal proteins to the cell surface, which may remain in the ER as aggregates. These results represent a first step towards a better understanding of the molecular mechanisms underlying impairment in cell-to-cell adhesion in Darier's disease.

181 [Oral 029]

BMP-4 Can Induce Keratinocyte Commitment by Inhibition of the Neural Antiapoptosis Pathway

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It has been stated that the differentiation of epidermis requires inductive signals while the neuralization of the dorsal ectoderm requires only an inhibition of this signaling, interpreted as a "default" state. BMP4 is a key regulator of this commitment since it can directly induce epidermal fate and inhibit the formation of neural tissue. Embryonic stem (ES) cell technology is an attractive model system for studying the initial molecular mechanisms underlying lineage differentiation. Under appropriate culture conditions, we show that these pluripotent cells can undergo efficient keratinocyte commitment. Furthermore, the ES-derived keratinocytes are able to reconstituted a pluristratified epidermis with a6β4 integrin expression to the basal layer and deposition of laminin-5. cDNA microarray technology was used for a search of genes whose steady-state levels are modulated during the early induction (2 h) by BMP-4. As confirmed by Real Time PCR, BMP-4 prominently repress neural embryonic-specific markers including genes encoding neural antiapoptotic ligands. BMP-4 activity has been identified as part of apoptotic mechanisms in many cell types *In vitro*. Therefore, we suggest that, as an active inducer of keratinocyte commitment, BMP-4 may interfere with the survival of neural precursor cells by inhibition of the neural antiapoptosis pathway.

182 [Oral 030]

Proteasome Inhibition Results in Trail Sensitization of Primary Keratinocytes by Removing the Resistance-Mediating Block of Effector Caspase Maturation

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TNF-related apoptosis-inducing ligand (TRAIL) exerts potent cytotoxic activity against transformed keratinocytes, whereas primary keratinocytes are relatively resistant. In several cell types, inhibition of the proteasome sensitizes for TRAIL-induced apoptosis by interference with NF-κB activation. Here we describe a novel intracellular mechanism for TRAIL-resistance of primary cells and how this resistance is removed by proteasome inhibitors. In contrast to findings in other cell types, retroviral infection of primary keratinocytes with IκBα (IκBα-TΔ) mutant or kinase dead IKK2 (IKK2-KD) dominant-negative mutants, potent inhibitors of NF-κB, did not result in modulation of TRAIL sensitivity, suggesting that sensitization by proteasome inhibition utilizes different signalling pathways. This sensitization was not mediated at the receptor-proximal level of TRAIL DISC formation or caspase 8 activation but further downstream. Activation of caspase 3 was critical as it only occured when mitochondrial apoptotic pathways, as reflected by Smac/DIABLO and cytochrome release, were activated. Smac/DIABLO is needed to release the XIAP-mediated block of full caspase 3 maturation. XIAP can effectively block caspase 3 maturation and, intriguingly, is highly expressed in primary but not in transformed keratinocytes. Our data suggest that breaking of this resistance via proteasome inhibitors, which are potential anticancer drugs, may sensitize primary keratinocytes to TRAIL-induced apoptosis and could complicate their clinical application.

183 [Oral 031]

Identification of a 4.2 kb 5′-Flanking Region of the Human Cdsn Gene That Directs Variable Expression in Hair Follicle and Epidermis of Transgenic Mice

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Corneodesmosin (Cdsn) is a protein secreted by the epidermis granular keratinocytes then incorporated into their desmosomes. Cdsn is also expressed in the inner root sheath (IRS) of human hair follicles. The homophilic adhesive properties of Cdsn reported *In vitro* argue for a role of the protein in intercellular cohesion. Transgenic mice bearing a 4.2-kb-long human genomic DNA fragment corresponding to the region located upstream the Cdsn translation initiation codon were generated. This fragment was obtained by screening a chromosome 6 genomic library (Max Planck-Institute of Molecular Genetic; Berlin) and sequenced. It was linked to the coding region of a nls-lacZ reporter gene. The mouse screening, performed by Southern blot and PCR, allowed identification of 4 independent founders. The reporter gene expression was searched by histoenzymology using X-gal as a b-galactosidase substrate in different skin sites (back, ventral, tail) as well as in five mouse organs (kidney, thymus, heart, spleen and liver). All mouse lines exhibited the same b-galactosidase expression pattern although some variations were observed in the expression level. The promoter activity was clearly detected in the nuclei of medulla cells of skin hair follicles. Furthermore, it was detectable in IRS Henley layer (lower follicle) and Huxley layer (upper follicle) cells, in new-born as well as in adult mice, but not in hair cortex cells. Surprisingly, we could not detect any enzymatic activities in the granular layer of the interfollicular epidermis, which is the main site of Cdsn expression in mice and humans. However, topical application of retinoic acid or phorbol 12-myristate 13-acetate (PMA) resulted in activation of the transgene expression in the epidermis parakeratotic cornified cells. Our results indicate that Cdsn expression in the interfollicular epidermis and the hair follicle may be controlled by different genomic sequences. The specificity of the transgene expression gives an interesting tool for driving protein to specific hair follicle compartments.

184 [Oral 037]

DCoH/HNF-1 Transcription of Human Tyrosinase

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Human melanocytes express the entire system for the de novo synthesis, recycling and regulation of 6 (R) L-erythro 5,6,7,8 tetrahydrobiopterin (6BH4) the essential cofactor/electron donor for the biosynthesis of L-tyrosine from L-phenylalanine via phenylalanine hydroxylase (PAH). Pterin 4a carbinolamine dehydratase (PCD) is the rate limiting enzyme for the recycling of 6BH4 and therefore this enzyme is an important control point for the supply of L-tyrosine for melanogenesis in melanocytes. In the melanocyte cytosol PCD exists as a tetramer, but its dissociation to a dimer produces the dimerisation catalyst (DCoH) for the transcription factor Hepatocyte Nuclear Factor 1a (HNF-1). Immunohistochemical examination of melanocytes *in situ* in the epidermis, and in cell cultures, establish colocalisation of PCD/DCoH and HNF-1in both the cytosol and the nucleus. These results show for the first time the presence of DCoH/HNF-1 transcription factor in the nucleus of melanocytes. The human tyrosinase gene promotor contains a 16-base inverted palindrome with sequence homology to the HNF-1 homodimer binding site (i.e. GTTAATATTCTAACCA) indicating a possible role for DCoH/HNF-1 in the transcription of the tyrosinase gene. A 439-bp fragment of the human tyrosinase promoter containing the DCoH/HNF-1 homodimer binding site was isolated from 3 individuals. All 3 DNA sequences were identical to the tyrosinase promoter sequence. Specific binding of pure DCoH/HNF-1 (recombinant proteins) to the tyrosinase promoter was conferred by band shift analyses. Since this binding domain is located 184 bases away from the TATA- box we can conclude a dual function for PCD/DCoH in the control of melanogenesis by melanocytes (a) by regulation the L-tyrosine supply via PAH and (b) by transcription of the tyrosinase gene.

185 [Oral 038]

Interactions of Human Myosin Va Isoforms are Tightly Regulated by the Tail Domain

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Myosin Va encoded by the dilute gene (d) in mice and the Griscelli locus in humans, is a dimer that through its mechanochemical N-terminal head domain facilitates movement of cargo along subcortical actin filaments. Primary human epidermal melanocytes express six endogenously myosin Va isoforms. It was already shown that isoforms containing exon F are most abundant in melanocytes, therefore we hypothesized that these isoforms probably have a melanocyte-specific function. To uncover the biological role of the six isoforms we introduced eGFP-myosin Va tail constructs in human melanocytes and melanoma cells. Using immuno-fluorescence confocal microscopy and immuno-electron microscopy, we found that exon F is absolutely required for myosin Va to interact with melanosomes in human melanocytes. Our yeast two hybrid screening and coimmunoprecipitation assays revealed a direct interaction of melanophilin and an indirect interaction of rab27a, with exon F transcripts. These data indicate that in human melanocytes rab27a serves as a myosin Va receptor on the melanosome membrane with melanophilin acting as a bridging molecule. Our data also shows that isoforms lacking exon F but containing exon D are suspected to associate with Golgi-derived vesicles. These results indicate that the myosin Va medial tail domain provides the globular tail domain with organelle-interacting specificity.

186 [Oral 039]

Characterization of a Human Melanocyte Transcript Coding for a New Protein, Mel-46.2, and the Hypothetical Brain Protein my038. Involvement in Melanocytic Cell Response and Resistance to UV-B Radiation

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cDNA microarray analysis of normal human melanocyte (NHM) transcripts modulated by a pseudo-physiological fluence of artificial ultraviolet UV-B radiation allowed for the selection of a strongly up-regulated Expressed Sequence Tag, EST46, that was characterized. EST46, was found expressed by RT-PCR at rather high levels in several melanoma cell lines but variably in melanoma tumors, suggesting a possible interaction with melanocarcinogenesis. EST46 sequence aligned with the 830 bp DNA sequence of the hypothetical brain protein my038 of unknown function. In NHM, Northern-blot experiments using an EST46-derived probe identified two transcripts of ~0.8 kb and ~1 kb. Sequencing of the EST46 clone spotted on the microarray showed that it included the previously reported my038 sequence with additional DNA stretches. Using the 5′-RACE methodology, we assessed the full-length sequence of the EST 46 related transcript, which corresponded to 1014 bp. We confirmed its presence in SK-MEL-2 melanoma cell line and in NHM. Entire EST46 mapped to chromosome 16 (16p12.2), within a region yet devoided of known Single Nucleotide Polymorphisms (SNP) and cytogenetic alterations. Two open reading frames (ORF) were borne by the full-length transcript. One coded for my038 (71 amino acids) and the other for a new melanocyte protein that we termed Mel-46.2 (51 amino acids). Similarly to my038, Mel-46.2 harbors three putative consensus protein sequence sites. Two are phosphorylation targets for protein kinases C and casein kinase II, whereas the other is a N-myristoylation site. Instrumental over-expression of my038 and Mel46.2 fusion proteins in SK-Mel-2 line using the new GATEWAY™ technology showed a cytoplasmic distribution. Importantly, functional studies ascribed a role for Mel-46.2 and my038 in melanocytic resistance to UVB radiation. In conclusion, we have characterized an unknown melanocyte transcript that responds to UV-B stress and that codes for both my038 protein and a new protein temed Mel-46.2Mel- which are involved in cell resistance to UVB, a risk factor for melanoma.

187 [Oral 041]

Functional Expression of CCR3 But Not of CCR7 in Lymphogenic Melanoma Metastasis

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Involvement of chemokines and their cognate receptors in tumor metastasis has been shown recently. In this study, we analyzed melanoma cells for the expression of CCR3. We examined lymph node metastases derived (n = 4) and CNS metastases derived melanoma cell lines (n = 4) as well as normal melanocytes (n = 3). CCR3 expression was detected in all the samples examined at the mRNA and protein level. Functional CCR3 was shown in all lymph node derived cell lines examined by means of migration and actin polymerization assays. CCR3 was not functional in CNS derived melanoma cell lines and normal melanocytes. We next looked at CCR7 expression which has recently been suggested to be involved in lymphatic metastasis. CCR7 expression was detected at the mRNA level but not on the protein level. No cell migration in direction of the CCR7 ligand 6Ckine could be observed when performing migration assays. Inhibition of G proteins and PI3 kinase abolished CCR3 mediated cell migration towards ist ligand eotaxin in lymph node derived tumor cells whereas MEK inhibition did not result in a reduced number of migrated cells. This suggests an involvement of the PI3 kinase pathway in CCR3 signaling in lymph node derived melanoma cell lines. To assess levels of CCR3 ligands eotaxin and RANTES, quantitative PCR was performed. mRNA amounts of these chemokines were highest in lymph nodes. Taken together, our data suggests that a functional PI3 kinase signaling pathway may lead to metastasis of melanoma cells expressing CCR3 to organs secreting high levels of the respective ligands.

188 [Oral 052]

$1\alpha,25(OH)_2D_3$ Inhibits IL-1α Induced NF-κB Activation and IL-8 Synthesis in Cultured Normal Human Keratinocytes by Increasing the IκB Expression

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IL-1α is an important inflammatory cytokine in the skin. It is expressed by the keratinocytes and has been suggested to play a key role in hyperproliferative and inflammatory skin diseases like psoriasis. IL-1α contributes to the activation of the nuclear transcription factor, NF-κB, which is an inducible enhancer of many inflammatory genes. NF-κB comprises a family of homo- and heterodimers of Rel proteins. The purpose of this present study was to determine the effect of $1\alpha,25(OH)_2D_3$ on the IL-1α induced NF-κB activation. NF-κB DNA binding activity was determined by EMSA using a consensus oligonucleotide containing the κB sequence from either the IL-8 or the p53 promotor. Cultured normal human keratinocytes were preincubated with $1\alpha,25(OH)_2D_3$ (10^{-7}–10^{-8} M) for 12 and 24 h before stimulated with IL-1α (10 ng per ml) for 15 min. When using the IL-8 consensus oligonucleotide a significant time dependent decrease in NF-κB DNA binding activity was seen with a 72% ± 12 and 65% ± 15 inhibition at 10^{-7} M and 10^{-8} M $1\alpha,25(OH)_2D_3$, respectively, after 24 h. Only insignificant changes were seen in NF-κB binding to the p53 consensus oligonucleotide under similar condidtions. As determined by ELISA the inhibition of NF-κB binding to the IL-8 oligonucleotide was parallelled by an inhibition of 48% ± 9 in the IL-1α induced IL-8 expression after incubation with $1\alpha,25(OH)_2D_3$ (10^{-7} M) for 24 h. Incubation of cultured normal human keratinocytes with $1\alpha,25(OH)_2D_3$ (10^{-7} M) for 1–48 h also lead to a time dependent increase in the expression of the NF-êB inhibitor, IκB, as determined by Western blotting. This increase became significant after 6 h and was maximal (105% ± 52) after 24 h of incubation. We conclude that $1\alpha,25(OH)_2D_3$ inhibits IL-1α induced NF-κB activcation and the resulting IL-8 expression in cultured human keratinocytes by increasing the IκB expresssion. Since no inhibition of NF-κB binding to the p53 oligonucleotide were seen, our findings indicate that $1\alpha,25(OH)_2D_3$ inhibits specific NF-κB homo- and heterodimers leading to decreased transcription of inflammatory genes.

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Eumelanin and Phaeomelanin Contents of Depigmented and Repigmented Skin of Vitiligo Patients

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Background: There are two chemically distinct types of melanin: the red–yellow phaeomelanin and the brown black eumelanin. Both types of melanin have been detected in human hair, epidermis and cultured melanocytes. Objectives: The cascade of events leading to disappearance/inactivation of melanocytes in pathogenesis of vitiligo is still not clear. The ratio of eumelanin and phaeomelanin have not been studied in vitiligo subjects. We conducted this preliminary study to quantify levels of both eumelanin and phaeomelanin in depigmented as well as repigmented patches of vitiligo on various treatments. Patients and Methods: We enrolled seven patients of vitiligo for this study. We took 3mm punch biopsies from either depigmented or repigmented skin of these patients except for two patients (RA and PI) in whom punch biopsies were taken both from repigmented as well as depigmented lesions. Two patients were on PUVA, one on PUVAsol, one on antioxidant, two on topical steroids and one untreated. The eumelanin and phaeomelanin contents of the skin biopsies were quantified by high performance liquid chromatography (HPLC) as described previously (Ito and Fujita 1985). Results: We analyzed melanin contents in five repigmented and four depigmented lesions. In five repigmented lesions average AHP/PTCA ratio was 0.85 (range 0.63–1.14) whereas in four depigmented lesions average ratio was 5.8 (range 3.36–9.95). Conclusions: Depigmented lesions showed predominantly phaeomelanin whereas repigmented lesions showed predominantly eumelanin. In complex sequence of events leading to death/inactivation of melanocyte, decreased tyrosinase activity or other factors leading to switch to phaeomelanin must be playing a vital role in pathogenesis of vitiligo. We could detect melanin in depigmented lesion of vitiligo of 5 years duration. It seems some residual melanocytes are still active in depigmented lesions.

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Hyperexpression of Extracellular Signal-Regulated Protein Kinase (ERK) 1/2 Mitogen-Activated Protein Kinase (MAPK) in Cultured Caucasian Melanocytes

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Variations in human pigmentation among different ethnic groups are due to differences in the amount and type of melanin produced in the epidermis. Considerable evidence supports a key role of melanocytes in determining the phenotypic difference between ethnic groups. The activity of the melanocyte-specific enzyme tyrosinase, which catalyzes the first step in the biosynthesis of melanin, is higher in melanocytes of black skin than those found in Caucasian skin. Recently, the MAPK signaling pathway has been implicated in the regulation of melanogenesis in B16 murine melanoma cell lines. Activation of MAPK in these cells inhibits melanogenesis. In addition, inhibition of MAPK signaling in human melanoma cells with either anthrax lethal toxin or MAPK inhibitors results in a dramatic increase in melanin production. We were interested in determining whether a correlation exists between activated MAPK and melanin production in melanocytes derived from Caucasian and Black donors. In immunoblot analyses using antibodies directed against activated MAPK we observed significantly more phosphorylated ERK 1/2 in the less pigmented Caucasian melanocytes than in Black melanocytes. Furthermore, westernblot analyses indicated significantly more hyperphosphorylated Rb in the Caucasian melanocytes than in the Black melanocytes. Rb is a negative cell cycle regulator, however, in its phosphorylated form it plays a key role in the transition from the G_1 to S phase of the cell cycle. This increased pRb is consistent with the observed higher proliferation rate of Caucasian melanocytes. Our data indicate that ERK activation in cultured human epidermal melanocytes is inversely proportional to melanogenesis.

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Expression of Ca^{2+} Binding Protein S100A4 (mts-1) in Normal Human Skin, Malignant Melanoma and Other Cutaneous Malignancies

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S100A4 (mts-1), a member of the S100 gene family, has been shown to be associated with cancer development and metastasis. We have evaluated the expression of S100A4 protein in melanocytic nevi (n = 38), primary cutaneous malignant melanomas (n = 39), cutaneous (n = 25) and lymph node (n = 15) metastases of malignant melanoma, squamous cell carcinomas (n = 4), basal cell carcinomas (n = 4), cutaneous T-cell lymphomas (n = 3), Kaposi sarcomas (n = 6), and Merkel cell carcinomas (n = 2) using a highly specific primary monoclonal antibody and immunohistochemical techniques (paraffin sections, conventional labeled streptavidin-biotin technique as well as laser scanning microscopy). In melanocytic tumors, staining was correlated with immunoreactivity for S100 and HMB-45 as well as with prognostic markers including tumor thickness and clark level. In contrast to previous observations, we found strong cytoplasmic and nuclear staining for S100A4 in melanocytic nevi, primary cutaneous malignant melanomas, cutaneous and lymph node metastases of malignant melanomas, T-cell lymphomas as well as Kaposi sarcomas, while S100A4 immunoreactivity was undetectable or very weak in basal cell carcinomas and squamous cell arcinomas. In S100A4-positive tumors, staining was in general focally pronounced in peripheral cells while central areas revealed reduced or no staining. In conclusion, we here report in contrast to previous observations strong immunoreactivity for S100A4 in melanocytic nevi, primary cutaneous malignant melanoma, cutaneous and lymph node metastases of malignant melanomas, T-cell lymphomas as well as Kaposi sarcomas. Our findings indicate that S100A4 expression may be of importance for the growth characteristics and metastatic potential of these malignant skin tumors, and the results encourage studies to evaluate the potential value of using S100A4 expression levels as markers in the clinical management of melanoma.

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Human Melanocytes Express β_2-Adrenoceptors *In Situ* and Produce Catecholamines

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Previously it was reported that human epidermal melanocytes neither express β_2-adrenoceptors nor produce catecholamines. However, we have demonstrated that cultured human epidermal melanocytes express tyrosine hydroxylase isozyme I mRNA (TH), the rate limiting enzyme in catecholamine biosynthesis. Subsequent RT-PCR and immunohistochemical analyses confirmed the expression of TH, dopa decarboxylase and dopamine-β-hydroxylase enzymes without the presence of phenylethanolamine-N-methyl-transferase (PNMT), the last enzyme in the catecholamine biosynthesis cascade. This result suggests that human melanocytes are noradrenergic cells. This data is supported by our previous finding of a time- dependant induction of α_1-adrenoceptors on melanocytes in response to noradrenaline, suggesting an autocrine regulation. By contrast human epidermal keratinocytes produce adrenaline. Here we report that human epidermal melanocytes also express β_2-adrenoceptors under *in vitro* conditions. Using the double-immuno-fluorescense technique for the β_2-adrenoceptor and HMB-45 for the detection of melanocytes in cryostat sections of human epidermis, high levels of β_2-adrenoceptors were found in melanocytes. Since adrenalin is a specific agonist for the β_2-adrenoceptor, yielding increased cAMP production in melanocytes, this adrenergic signal could regulate *de novo* pigmentation via the β_2-adrenoceptor/cAMP cascade. This paracrine control supports the symbiotic relationship existing between melanocytes and keratinocytes in the human epidermis.

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Bcl-2 Independent Induction of Apoptosis by Bisphosphonates in Human Melanoma Cells

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Bisphosphonates are synthetic pyrophosphate-analogues, well established in the treatment of osteoclast-mediated resorbtive bone diseases, including osteoporosis, Paget's disease of bone and tumour induced osteolysis. Recent studies suggest that, beside inhibiting bone resorbtion, bisphosphonates may also exert a direct antitumour effect, and this class of drugs has been shown to inhibit proliferation and to induce apoptosis *In vitro* in different human tumour cell lines. In the case of the nitrogen-containing subclass of bisphosphonates, this antineoplastic activity could be related to the inhibition of the mevalonate pathway, and consequently, of the prenylation of signalling proteins such as small GTPases. We examined the *In vitro* effect of nitrogen containing bisphosphonates pamidronate and zoledronate, as well as of the nonamino bisphosphonate clodronate on melanoma cell lines A 375, M186 and on the bcl2 overexpressing melanoma cell line A375/Bcl2. Apoptosis, proliferation and cell cycle changes were assesed. We could show that bisphosphonates are able to induce apoptosis, to inhibit cell growth and to induce S-phase changes of the cell cycle in melanoma cell lines, in dose dependent manner. The intensity of these antitumoral effects did not correlate well with the relative antiresorbtive potency of the bisphosphonates. Interestingly, the susceptibility of melanoma cells to bisphosphonates-induced apoptosis was not influenced by bcl2 overexpression. Our results indicate for the first time that bisphosphonates have a direct antitumour effect on melanoma *In vitro* and suggest that these substances could play a role in the adjuvant therapy of melanoma in the future.

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Normal Human Epidermal Keratinocytes (NHEK): Active Influx Transport is Mediated by Members of the Organic Anion Transport Polypeptide Family

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Normal human epidermal keratinocytes (NHEK) have been shown to express a cell type specific pattern of extrahepatic cytochrome P450 enzymes and efflux transport proteins indicating the ability of keratinocytes to metabolize and excrete a variety of xenobiotics. Recently transport proteins involved in the uptake of xenobiotics have been detected and here we analysed the mRNA- and protein- expression profiles and functional activities of these proteins in keratinocytes in comparison to primary liver cells. The transporters studied included the subtypes A, B, C, D and E of the organic anion transporting polypeptide (OATP) family which are responsible for the uptake of various anionic and neutral molecules and especially organic cations including drugs. In our study we were able to show a constitutive expression of OATP -B, -D and -E in normal epidermal keratinocytes using RT-PCR and Northern blot analysis, as well as in human skin tissue shown by tissue blot hybridization and immunostaining. Expression of OATP-A and -C was not detected in any of the keratinocyte samples. In contrast, liver tissue showed a significant expression of OATP-A and -B as well as OATP-C, a weak expression of OATP-D and no expression of OATP-E. These data revealed that human epidermal keratinocytes express a specific profile of transporters involved in drug influx. In addition using a newly developed uptake-transport assay, we were able to show, that uptake of known OATP substrates like ^{3}H-estradiol-17β-glucuronide in NHEKs can be inhibited by specific inhibitors like taurocholate, proving the functional activity of the expressed OATPs. Even though the substrate specificity of the OATP isoforms is only partially known till now, our findings give strong evidence that the uptake of large organic cations like drugs in keratinocytes is an active transport process mediated by members of the OATP family. *In vitro* this process can be modulated by specific competitive inhibitors.

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Altered Gene Expression in Normal Human Epidermal Keratinocytes (NHEK) by Retinoic Acid (RA) Isomers and Their 4-oxo-Metabolites

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Vitamin A and its natural and synthetic derivatives (retinoids) exert profound effects on fundamental processes such as vision, embryonic development and cell proliferation and differentiation. Retinoids are effective in the treatment of numerous dermatologic disorders. There is increasing evidence that target tissue metabolic transformation is crucial for regulation of their biological and pharmacological activity. In this experiment RA isomers and their 4-oxo metabolites were incubated with cultured NHEK for 24 h and analysis of differentially expressed genes was performed using DNA-Chip-technology. We examined 4400 sequence-validated cDNAs and ESTs arrayed on DermArray GeneFilters microarray and analyzed suitably exposed autoradiograms with Pathway analysis software. Incubation of cells with all-*trans*-RA for 24 h revealed up-regulation of the human metallothionein (MT)I-F gene, corticotropin releasing hormone-binding protein, glutathione S-transferase M5, epidermal growth factor, keratins 8 and 13 and interleukin 18 receptor. Genes encoding for keratin 10, human interleukin enhancer binding factor 3, steroid-5-α-reductase and CYP2C8 were down-regulated simultaneously. Incubation with 4-oxo-all-*trans*-RA showed a different gene expression profile with up-regulation of keratin 15, thiosulfate sulfurtransferase, fibroblast growth factor receptor 2, δ-aminolevulinate-synthase 1, CYP3A3 and TIMP3. In contrast 9-*cis*- and 4-oxo-9-*cis* RA as well as 13-*cis*-RA and 4-oxo-13-*cis* RA revealed different expression profiles. Genearray analysis is a powerful tool for exploring retinoid effects in the skin.

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Defective Trafficking and Cell Death are Characteristic of Skin Disease-Associated Connexin 31 Mutations in Keratinocytes

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Distinct germline mutations in the gene encoding connexin 31 (Cx31) underlie the skin disease, Erythrokeratoderma variabilis (EKV) or sensorineural hearing loss with/without peripheral neuropathy. Here we describe a number of functional analyses to investigate the effect of these different disease-associated Cx31 mutants on connexon trafficking and intercellular communication. Immunostaining of a skin biopsy taken from an EKV patient harbouring the R42P mutation revealed sparse epidermal staining of Cx31, and, when present, it had a perinuclear localisation. Transfection and microinjection studies in both keratinocytes and fibroblast cell lines also demonstrated that R42P and three other EKV-associated mutant Cx31 proteins displayed defective trafficking to the plasma membrane, being either retained in the Golgi apparatus or in the cytoplasm. However, the defective trafficking was not shown in nonepidermal cells such as HeLa, with some gap junction plaque formation. In contrast, the deafness/neuropathy mutant 66delD and R32W, a variant with no identified disease association, could traffick to the plasma membrane but did not form gap junction plaques and were unable to dye-transfer. Another interesting characteristic feature observed with the dominant skin-disease Cx31 mutations was a high incidence of blebbing and cell death. This was not observed with wildtype, 66delD or R32W Cx31 proteins. In conclusion, we have identified some key phenotypic differences with respect to different disease-associated Cx31 mutations. The mechanism of cell death associated with the EKV Cx31 mutation is currently under investigation.

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Characterization of a First Domain of Human High Tyrosine/Glycine and High Sulfur Keratin Associated Protein (Kap) Genes on Chromosome 21q22.1

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Analysis of the EMBL/Genebank database using nonhuman keratin associated protein (KAP) cDNA sequences as a query resulted in the identification of a first domain of high tyrosine-glycine and high sulfur KAP genes located on human chromosome 21q22.1. This domain, present on BAC clones AP001078 and AP001709, was *c.* 535 kb in size and contained 17 high tyrosine-glycine- and 7 high sulfur KAP genes, as well as 9 KAP pseudogenes. These genes could be divided into 11 families (the KAP6-8, 11, 13, 15, and 19–23 families) based on amino acid homology comparisons of the putative KAP gene open reading frames. Systematic cDNA isolation and *in situ* hybridization expression studies of all of the KAP genes identified in this region showed varying degrees of expression of 12 members of the high tyrosine/glycine- and 6 members of the high sulfur KAP genes in the differentiating portions of the hair fiber cortex and cuticle.

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Diverse Effects of Interleukin-1 on Apoptosis Induced by Ultraviolet B Radiation

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Irradiation of cells with ultraviolet B (UVB) induces apoptotic cell death by triggering at least two different and independent pathways. On the one hand, UVB induces genomic DNA damage, on the other hand, it causes activation of membrane bound cell death receptors like CD95/Fas or the TRAIL-receptors. Interleukin (IL)-1 was shown to prevent the epithelial cell line KB from undergoing apoptosis induced by the death ligands CD95L or TRAIL. In contrast, UVB-induced apoptosis was not only not prevented, but was even enhanced upon pretreatment with IL-1. It was the aim of the study to elucidate the mechanism responsible for IL-1 to act once anti- and once proapoptotic. Western blot analysis revealed that prestimulation of KB cells with IL-1 leads to a NFκB-dependent up-regulation of the inhibitor of apoptosis proteins (IAP), c-IAP and x-IAP, which ultimately rescues cells from CD95L- or TRAIL-induced apoptosis. In contrast, stimulation of cells with IL-1 15 min prior to UVB exposure induces the proapoptotic cytokine tumor necrosis factor-α (TNFα). This up-regulation is also dependent on NFkB. Time course studies revealed that the temporal sequence of the addition of IL-1 and the apoptotic stimulus critically determines whether NFκB targets the IAPs or TNFα. Addition of IL-1 8–6 h prior to UVB results in up-regulation of IAPs and reduction of UVB-induced apoptosis. The closer IL-1 prestimulation gets to UVB irradiation (120–15 min), the stronger apoptosis is induced and this coincides with an increase of TNFα release. The same applies when IL-1 is added after UVB irradiation. IL-1 given 15–60 min after UVB results in enhancement of apoptosis and increased release of TNFα, whereas later applications of IL-1 do not affect UVB-induced apoptosis. Taken together, the study demonstrates that the anti/proapoptotic activity of a stimulus does not only depend on its nature and on the stimulus causing apoptosis but also on the temporal sequence when the stimulus is applied.

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Biological Effects of Sphingosine 1-Phosphate on Keratinocytes

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Sphingosine 1-phosphate (S1P), which is a lipid mediator, is stored in platelets, and is released upon their activation. S1P has potent biological activities such as effects on cell proliferation, differentiation and cell motility for endothelial cells and melanoma cells. Although it has been reported recently that S1P also has an effect on cutaneous wound healing of diabetic model mice, the biological effects of S1P on keratinocytes have not yet been investigated. In this study, we examined not only the biological effects of S1P on keratinocytes but also its physiological effects on the skin. RT-PCR analysis of S1P receptors revealed that human keratinocytes express edg–3 and edg–5 mRNAs, which are transcripts for S1P receptors. Addition of S1P to cultured keratinocytes raised the concentration of intracellular calcium within 1 min, while the growth of keratinocytes was not affected. An *In vitro* scratch assay, which evaluates cell mobility, showed that S1P has an inhibitory effect on keratinocyte motility in the presence of bovine pituitary extract. When keratinocytes were seeded on collagen gels and the diameter of the gel was measured 1 h after removal from the plate, keratinocyte contractile ability was augmented in the presence of S1P. S1P was also applied once daily to 6 mm-diameter full thickness wounds of normal rat dorsal skin. Comparing the relative wound areas 6 days later, treatment with S1P accelerated wound healing. These findings suggest that S1P has various biological effects on keratinocytes via its specific receptors and on the physiological function of the skin, such as the promotion of cutaneous wound healing.

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Corticotropin Releasing Hormone (CRH): An Autocrine Hormone That Promotes Lipogenesis in Human Sebocytes

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Sebaceous glands may be involved in a pathway conceptually similar to that of the hypothalamic-pituitary-adrenal (HPA) axis. Such a pathway has been described and may occur in human skin and in the sebaceous glands since they express neuro-peptide receptors. CRH is the most proximal element of the HPA axis and it acts as central coordinator for neuroendocrine and behavioral responses to stress. To further examine the probability of a HPA equivalent pathway we investigated the expression of CRH, CRH-binding protein (CRH-BP) and CRH receptors (CRH-R) in SZ95 sebocytes *In vitro* and their regulation by CRH and several other hormones. CRH, CRH-BP, CRH-R1 and CRH-R2 were detectable in SZ95 sebocytes at the mRNA and protein levels: CRH-R1 was the predominant type (CRH-R1/CRH-R2 = 2). CRH was biologically active on human sebocytes: it induced biphasic increase in synthesis of sebaceous lipids with a maximum stimulation at 10^{-7} M and up-regulated mRNA levels of 3b-hydroxysteroid dehydrogenase/D^{5-4} isomerase, although it did not affect cell viability, cell proliferation or IL1b-induced IL-8 release. CRH, dehydroepiandrosterone and 17b-estradiol did not modulate CRH-R expression, whereas testosterone at 10^{-7} M down-regulated CRH-R1 and CRH-R2 mRNA expression at 6–24 h and GH switched CRH-R1 mRNA expression to CRH-R2 at 24 h. Based on these findings, CRH may be an autocrine hormone for human sebocytes that exerts homeostatic lipogenic activity, whereas testosterone and GH induce CRH negative feedback. The findings implicate CRH in the clinical development of acne, seborrhea, androgenetic alopecia, skin aging, xerosis and other skin disorders associated with alterations in formation of sebaceous lipids.

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Characterization of the Protein Responsible for Mal de Meleda

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Mal de Meleda (MDM) (OMIM 284300) was originally described in patients from the island of Meleda, Croatia. This disease is inherited in an autosomal recessive manner with an estimated frequency of 1:10^5 in the general population. Mal de Meleda is characterized clinically by an inflammatory palmoplantar keratoderma and transgressive pachyderma mainly involving palms and soles. Histological features include acanthosis, hyperkeratosis and pseudospongiosis. The MDM gene was within a cluster of Ly-6 homologous genes, which are members of the Ly-6/uPAR family of receptor and secreted proteins. Sequence analysis of patients suffering from MDM revealed three different homozygous mutations in the *ARS* (*component B*) gene, encoding SLURP-1 (*S*ecreted *Ly*-6/*u*PAR *R*elated *P*rotein 1). Expression analysis of SLURP-1 transcripts by Northern blot and RT-PCR reavealed that SLURP-1 is mainly expressed in plantar, and most likely, in palmar skin, which corresponds to the sites of MDM lesions. SLURP-1 has homologies with the uPAR (*u*rokinase *P*lasminogen Activator *R*eceptor) family, which acts as a scaffolding protein of integrins, but does not contain a GPI membrane anchor. Furthermore, SLURP-1 has homologies with snake toxins. These properties will serve as candidate avenues to explore the function of SLURP-1, which is likely to function as a ligand for a receptor yet to be identified. To this end, a myc-tagged SLURP-1 cDNA has been cloned in p-Bud. Both palmoplantar and nonpalmoplantar keratinocytes are transfected by SLURP-1; protein expression and localization is monitored by immunofluorescence and immunoblot analysis. Recombinant myc-tagged SLURP-1 protein was obtained after Ni-NTA purification from transfected 293T cells culture medium, indicating that SLURP-1 is a secreted protein. The recombinant protein is used for the identification and molecular characterization of the binding partner of SLURP-1.

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Transcriptional Effect of a Mallow Extract in Human Reconstituted Epidermis Using cDNA Macro-Array

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Many clinical studies reported the positive effect of *all-trans* retinoic acid topical application on photo-damaged skin and on wrinkles. Thus, in order to investigate the antiageing activities of a mallow flower extract, we studied the modifications of the gene expression patterns induced by this extract, vs. *all-trans* retinoic acid (RA), in a human epidermis model (RHE). The mRNA expression profiles were studied using a customized cDNA macro-array system containing 598 skin-related genes. Differentiated RHE were treated with the *Malva sylvestris* extract (3%) or with RA (1 mM) dissolved in culture media for 8 h or 24 h. After RHE lysis, mRNAs were isolated, reverse-transcribed, ^{33}P-labelled and the probes obtained were hybridized to the cDNAs on the membranes and revealed by phosphorimaging. Qualitative analysis from all RHE showed that more than 25% of the selected genes were significantly detected on the array. No significant difference in the gene expression profiles was observed when untreated RHE were analysed after 8 or 24 h of incubation. According to the nature and the length of the treatments, more than 40 genes were found to be modified. Comparison of the expression profiles after 24 h treatments with RA or *Malva sylvestris* extract showed differences in only 15% of genes. We observed that genes previously reported to be modulated by RA were also regulated by the *Malva sylvestris* extract treatment (cytokeratin 10, 2E, calgranulin A and B, IL-1RA,…). These results show that the *Malva sylvestris* extract, studied in the human reconstructed epidermis model, induces a gene expression profile very similar to that observed with a known antiwrinkle molecule.

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Effects of Recombinant Overexpression of PKCα and δ on Receptor-Coupled Calcium Handling in HaCaT Keratinocytes

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We have previously shown that the modification of protein kinase C (PKC) activity by forbol esters affects the kinetic properties of the purinergic receptor-mediated calcium response in HaCaT keratinocytes. In the present study we investigated the features of certain (purinergic, bradykininergic) receptor-coupled processes that result in an increase of intracellular calcium concentration ($[Ca^{2+}]_i$) on keratinocytes which stably overexpress either the classic α or the novel δ isoform of PKC. The clones were found to have essentially the same resting $[Ca^{2+}]_i$ (65.4 ± 2.4 nM in α and 71.3 ± 2.3 nM in δ clones) but responded with larger calcium transients to the application of 180 μM ATP (223 ± 18 for α and 238 ± 21 nM for δ clones vs. 156 ± 18 nM in control cells). Examining the declining phase of the calcium signals revealed a 37.4 ± 7.3 s time constant for calcium removal in control and 39.8 ± 7.1 s in PKCα overexpressing cells. However, PKCδ clones displayed a prolonged falling phase characterised by a 63 ± 4.8 s time constant. While ATP was effective on all cells examined, including both clones, bradykinin (20 μM) failed to induce a calcium transient in 50% of control cells. The responsiveness to bradykinin was greatly suppressed in PKCα and PKCδ overexpressing keratinocytes (less than 20%). However, if the given cell did respond to the drug, the evoked calcium signal displayed all characteristics described for those induced by ATP, namely, the removal of calcium from the cytoplasm was slower in PKCδ clones. Unlike the ATP induced elevation of $[Ca^{2+}]_i$, bradykinin evoked signals tended to decline even in the continous presence of the drug indicating a faster desensitisation of the bradykininerg pathway. In this respect the clones did not differ significantly from control cells. Together with changes in calcium handling, the stable overexpression of these PKC isoforms altered the proliferation and differentiation properties of the cells. Both the PKCα and δ clones displayed decreased proliferation tendencies and increased expressions of the late diferentiation marker involucrin. These observations argue that certain PKC isoenzymes play crucial roles in the regulation of calcium handling and differentiation of keratinocytes.

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Stretch-Activated Channels and Their Regulation by Protein Kinase C in HaCaT Keratinocytes

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The skin is almost continuosly under mechanical stress. Certain areas observe increased pressures while others are stretched, as the marginal cells of the wound bed. These mechanical forces are believed to induce proliferation and/or differentiation of keratinocytes. Here we demonstrate the presence and their regulation by protein kinase C (PKC) of stretch-activated channels in the human keratinocyte cell line HaCaT in culture. Cells were voltage clamped using the whole-cell patch-clamp technique, changes in intracellular calcium concentration were assessed using the fluorescent calcium probe fura-2. Alterations in the membrane potential were followed using a conventional microelectrode. The activation of these cells by hypotonic stress, a drop in osmolarity to 75% and 50%, caused hyperpolarisation, by 15.2 and 39.7 mV, respectively, from a resting value of –28.4 ± 4.8 mV. The changes were readily reversible. Similar alterations in the membrane potential were observed by changing the hydrostatic pressure. An overnight incubation with 100 nM phorbol 12-miristate 13-acetate (PMA), which induced a down-regulation of PKC isoforms, caused a pronounced hyperpolarisation of these cells by 16 ± 3.2 mV, but did not affect their ability to respond to the hypotonic challenge. Consistent with these findings, the recombinant overexpression of PKCα resulted in a depolarization of the membrane potential (to –20.1 ± 2.5 mV). Hypotonic solutions caused a small increase in intracellular calcium concentration, from a resting value of 77 ± 4–124 ± 16 nM and from 72 ± 7 to 118 ± 24 nM in 50% and 75% hypotonic solution, respectively. However, the development of these changes and the return to the initial level was much slower than those evoked by ATP. The pretreatment with PMA did not influence the calcium transients evoked by hypotonic solutions. The intracellular calcium concentration increase was 57 ± 13 nM in 50% and 43 ± 11 nM in 75% hypotonic solution in pretreated cells. These findings connect the observed changes in voltage following mechanical stress to the activation of Cl^- and probably to Ca^{2+}-activated potassium channels. Furthermore, the regulation of the above process by certain PKC isoforms is probable.

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Mutations of Connexin 31 and 30.3 in Erythrokeratodermia Variabilis

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Gap junctions are clusters of intercellular channels connecting the cytoplasm of neighbouring cells. The subunits of the channels are formed by the connexins (Cx), proteins containing four transmembrane domains. Functionally, gap junctions allow rapid transfer of ions and small second messenger molecules, of less than 1 kDa in size, between adjacent cells. Therefore they permit coordinated response of groups of cells to external stimuli. This cell-cell communication is crucial for growth control and differentiation, as well as for maintaining tissue homeostasis. Each tissue expresses a specific subset of connexins. In skin, at least 9 connexins are expressed, including Cx31 and Cx30.3. Mutations in either Cx31 or Cx30.3 have been described to be causally involved in erythrokeratodermia variabilis (EKV). Erythrokeratodermia variabilis is a genetically heterogeneous disorder of cornification, transmitted in an autosomal dominant manner and caracterised by fixed patches of hyperkeratosis and migrating erythematous areas. The specific role of each of these connexins in the physiology and pathophysiology of epidermal tissues and the clinical phenotype of connexin mutants have not been elucidated yet. Cx31 and Cx30.3 coding regions have been subcloned in different combination in a vector designed to drive simultaneously constitutive, high-level expression of two transgenes in order to test the capacity of mutant and wild type Cx30.3 to form active channel. Expression, trafficking, localisation and interaction of the proteins are studied in communication-deficient HeLa cells. In transfected cell, wild type Cx31 and Cx30.3 colocalised at plasma membrane especially at cell-cell contact and it appears that they interact with cytoskeletal proteins. The intercellular coupling of Cxs is studied by examining the transfer of Lucifer yellow between stable HeLa cell lines expressing Cx31 and wild type or mutant Cx30.3.

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Characterisation and Function of Novel EDC Genes

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The epidermal differentiation complex (EDC) on human chromosome 1q21 contains three clustered families of genes encoding: (1) precursor proteins of the cornified cell envelope (2) S100 proteins and (3) the fused gene family combining characteristics of both. In this study we searched for novel genes on 1q21 expressed in the epidermis. We identified in silico a new member of the ''fused'' subgroup of S100 proteins (TRP). TRP transcripts were detected by RT-PCR in fetal bladder, scalp, foreskin, and in cultured primary keratinocytes. Analysis of a contig spanning ~680 kbp from human chromosome 1q21 allowed to identify several XP5 homologues as well as the complete XP32 and XP33 genes which differ from previously published incomplete sequences. Using XP5a and XP32 probes for analyzing a wide range of human tissues by RT-PCR and Northern blotting, we found high levels of transcripts in scalp and foreskin. XP33 RNA was detected in cultured keratinocytes, adult kidney, scalp skin, foreskin, and fetal bladder. In order to gain further insights into the functions of TRP and XP genes, coding sequences of the TRP and XP genes were cloned in frame to GST in the bacterial expression vector, pGEX-4T-1. Recombinant proteins have been purified from bacterial lysates using Glutathione Sepharose 4B affinity chromatography. Polyclonal rabbit antibodies against peptides of human XP5a, XP32, XP33 and TRP have been developed and are affinity purified. Antibody specificities are tested by Western blot analyses of bacterially expressed GST-fusion proteins. To investigate the protein distribution and subcellular localization of these proteins, fusions between GFP and the TRP, XP5, XP32 and XP33 coding sequences are constructed. HEK293T and HaCat cells transfected with these constructions are analysed by immunofluorescence. In future experiments we will investigate the differential expression of the XP family members, as well as TRP by tissue microarrays using antibodies and specific *in situ* probes.

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BP180 Autoantibody Detection in Bullous Pemphigoid Sera: Improved Sensitivity of a NC16A ELISA with Additional BP180 Selected Epitopes

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The bullous pemphigoid (BP) antigen BP180 contains an immunodominant region, NC16A, mapped within the membrane-proximal non collagenous stretch of the extracellular domain. ELISAs with NC16A domain have been recently developed as a diagnostic tool for BP autoantibody detection. Since BP180 autoantibody reactivity is not restricted to NC16A, we have investigated the possibility to develop an ELISA based on selected epitopes in addition to NC16A. At first, a glutathione-S-transferase (GST) -NC16A ELISA was set up and used to test the reactivity of 77 BP sera. Autoantibodies against the NC16A domain were detected in 62 of 77 BP sera (81%), with a sensitivity similar to that of previously described BP180 ELISAs. In order to assess if the 15 BP sera negative in our NC16A ELISA recognised other antigenic sites on the BP180 molecule, we have then analysed their reactivity against 16 BP180 epitopes exposed on l bacteriophage. These epitopes had been previously affinity selected from a BP180 random epitope l library using BP sera. The epitopes were transferred on nitrocellulose filters and used as targets in the immunological screening procedure with the 15 BP sera negative in NC16A ELISA. 5 and 3 of 15 sera recognised the C-terminus (AA 1331–1404) and the mid-portion of BP180 ectodomain (AA 1080–1107), respectively. Therefore, two additional GST fusion proteins (GST-1080 and –1331) were produced and used to set up an ELISA system with three recombinant proteins (GST-NC16A, –1080- and –1331). Using this approach 69 of our 77 BP sera resulted positive, with an increase in sensitivity from 81% to 90%. Our ELISA based on selected BP180 epitopes is not only more sensitive but also more informative of BP sera epitope pattern than ELISA using GST-NC16A fusion protein alone. Furthermore, these results show that the epitope mapping strategy represents a powerful tool to select epitopes to be exploited for diagnostic purposes.

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Induction of Connexins Cx30 and Cx26 in Epidermis Adjacent to Malignant Melanoma but Not to Melanocytic Nevi

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Communication between cells is performed by exchange of small molecules through gap junctions. Gap junctions connect adjacent cells and form communicating channels. They consist of two connexons (one of each cell), each formed by six transmembrane proteins called connexins (Cx). Ten of the known 21 different connexins are expressed in human skin. Gap junctions formed by different connexins are selective for different small molecules (e.g. second messengers). Thereby they control the specificity of cell-cell-communication. There is evidence suggesting that connexins play a role in tumour biology. This work concerns the expression of Cx30 and Cx26 in malignant melanoma and melanocytic nevi and in epidermis adjacent to these lesions. Investigations were performed by immunofluorescence microscopy using previously described specific antibodies. As previously shown Cx30 and Cx26 are only weakly, if at all, expressed in normal interfollicular epidermis. Now we show that both proteins neither occur in malignant melanoma nor in melanocytic nevi. In contrast both Cx30 and Cx26 are expressed in the epidermis adjacent to malignant melanoma but not to melanocytic nevi. These results clearly show an induction of Cx30 and Cx26 in normal interfollicular epidermis adjacent to malignant melanoma. There is no such induction in benign melanocytic nevi. Previously we demonstrated similar results investigating gap junction proteins in malignant epithelial skin tumours. So there is evidence that the change of the composition of gap junctions in the epidermis adjacent to malignant skin tumours may play a role in the metastasation of these tumours. Ongoing investigations will elucidate the function of Cx30 and Cx26 in normal interfollicular epidermis adjacent to malignant skin tumours.

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Characterisation of Keratinocytes Upon Low-Dose Acetaldehyde Exposure

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Acetaldehyde (AAL) is an indoor pollutant, used for the production of desinfectants and plastic materials, and therefore might effect the skin by direct or aerogen contact. Furthermore, AAL is synthesised during alcohol metabolism. So far no specific data are available concerning the effects on human keratinocytes. Therefore we studied effects of low-dose concentrations of AAL on cytoskeleton and proliferation. Normal human epidermal keratinocytes were cultured under serum-free conditions. Cells were exposed for eight hours to variable concentrations of acetaldehyde (0.01, 10, 1000 μM). Differentiation and proliferation associated Cytokeratines (CK) 1, CK 10, CK 6, CK 16 and the marker of mitosis MIB-1 were labeled immunocytochemically using monoclonal antibodies. Additionally, proliferation was quantified fluorometrically using the alamarBlue assay. The synthesis of CK 1, CK 10, and CK 6 were not affected by AAL, whereas the number of CK 16 positive cells were 25% higher than controls. MIB-1 was not altered by AAL. Using the alamarBlue-assay, a high dose of acetaldehyde (1000 μM) caused an inhibition of 66 ± 8,7%, an intermediate dose (10 μM) caused no change, whereas a low dose (0.01 μM) increased the proliferation of keratinocytes by 24 ± 3,1%, compared to controls. In contrast to high doses of AAL, which most likely cause cytotoxicity, low doses of AAL induced keratinocyte proliferation. AAL might be considered as cofactor for proliferative, possibly precancerous, processes in skin.

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Caspase-14 Expression is Regulated by Cooperation of Transactivating and Repressing Factors During Keratinocyte Terminal Differentiation

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Caspase-14, a recently identified member of the caspase family of proapoptotic proteases is thought to be involved in epidermal differentiation. Caspase-14 expression is highly restricted to suprabasal layers of the epidermis. In order to understand the regulation of this protease, we investigated factors controlling keratinocyte specific caspase-14 gene-expression. Deletion mapping of the 5′-region of caspase-14 revealed a minimal promoter sequence of ~270 bp relative to the start sites of transcription. This minimal promoter element contains two potential AP-1 sites (AP-1.2, AP-1.3) three potential AP-2 binding sites and one potential NFkB site. Transfection experiments and electrophoretic mobility shift assays encompassing these transcription factor binding sites and their mutated counterparts showed that caspase-14 AP1.3 and NFkB sites were functional in keratinocytes. Caspase-14 promoter constructs showed higher activity in postconfluent keratinocytes as compared to preconfluent cells. This finding demonstrates that keratinocyte differentiation is indeed a determining factor for caspase 14 promoter activity. In addition we identified three functional repressor sites (f1/2, f5/6, f6/8) in the caspase 14 promoter region. One of them (f5/6) contains a PAUSE-1 recognition sequence, a silencer element recently identified in the plasminogen activator inhibitor (PAI-2) gene, which has an identical expression pattern as caspase-14 in the epidermis. Our data demonstrate that the specific expression of caspase-14 in the suprabasal layers of the epidermis is regulated by enhancing (AP-1, NFκB) as well as silencing (PAUSE-1) regulatory elements. Modulation of these transcription factors therefore is likely to have an effect on caspase 14 expression and represents a potential strategy for influencing epidermal barrier formation.

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Ceramide C-2 and Hydrogen Peroxide Induce Apoptosis by Two Different Pathways in Human Keratinocytes

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Apoptosis is a physiologic programmed cell death (PCD) that occurs throughout the epidermis. It contributes to epidermal protection and maintenance by removing damaged or unwanted cells averting inflammation and tissu alterations. Ceramides, synthesized after activation of the sphingomyelin cycle, have been shown to be the major second messengers of this process and to be produced by oxidative stress. To better understand intracellular events linked to oxidative stress-induced PCD, the induction of apoptosis was carried out by comparing ceramide C-2 and hydrogen peroxide both on HaCaT cells, a human keratinocyte cell line mutated on both alleles of the p53 gene, and normal human keratinocytes (NHK). Apoptosis signalling was investigated by measuring caspases-1, –3, –8, –9 activities, mitochondrial transmembrane potential (fluorescent probe, JC-1), and plasmic membrane integrity (fluorescent probe, Toto-3). For NHK and HaCaT cells, the mitochondrial transmembrane potential ($\Delta\psi_\mu$) was not affected by ceramide C-2 treatment, while a collapse in $\Delta\psi_\mu$ was induced by H_2O_2 in both cell types. This phenomenon was associated with a dramatic increase of caspase-8 and, to a smaller extent, of caspases-9 and –3 activities. In conclusion, signalling pathways induced by ceramide C-2 or hydrogen peroxide are based on two distinct mechanisms. Moreover, the mutated p53 of the HaCaT cells, do not affect their response to the biologically active lipid C-2 and to H_2O_2 compared to normal cells.

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The Low-Affinity Neurotrophin Receptor p75 Mediates the Proapoptotic Activity of Brain-Derived Neurotrophic Factor in Keratinocytes

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The low-affinity neurotrophin (NT) receptor p75 is a member of the Fas/tumor necrosis factor (TNF) receptor family which mediates cell death. The NT brain-derived neurotrophic factor (BDNF) is synthesized by human keratinocytes, while these cells express p75 but not the BDNF high-affinity receptor trkB. The aim of the present study was to elucidate the role of p75 in cultured keratinocytes. BDNF dose-dependently decreased the number of keratinocytes, as shown by crystal violet staining ($p < 0.05$). Moreover, BDNF dose-dependently induced keratinocyte apoptosis, as shown by MTT assay ($p < 0.05$). BDNF also increased the rate of apoptosis induced by the trk inhibitor K252. As keratinocytes do not express trkB, it is likely that BDNF activities are mediated through p75. Indeed, antip75 neutralizing antibody alone induced keratinocyte apoptosis, as compared to controls. Secondly, simultaneous addition of BDNF and antip75 resulted in a rate of apoptosis similar to that of antip75 alone. In order to support the proapoptotic role of p75, parental (SK-N-BE) or p75 transfected (SK-N-BE p75) neuroblastoma cell lines were used. While BDNF did not induce apoptosis in SK-N-BE cells, it caused cell death in SK-N-BE p75, as shown by TUNEL. Finally, UVB-induced apoptosis was markedly increased in SK-N-BE p75, as compared to parental cell line. These results indicate that BDNF acts as a proapoptotic NT in human keratinocytes through p75, even in the presence of other trk receptors. Moreover, p75 seems to play a role in UVB-induced apoptosis. Taken together, these data confirm the proapoptotic role of p75 and represent the first functional evidence of p75 acting as a death receptor in keratinocytes.

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Distinct Isoforms of CD1d are Expressed in Keratinocytes in a Proliferation-Differentiation Dependent Manner

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CD1d is a nonclassical antigen-presenting molecule capable to present glycolipid antigens to NKT cells, which have been suggested to play a role in the pathogenesis of psoriasis. There are conflicting results concerning CD1d expression in keratinocytes. The aim of the present study was to investigate the expression of CD1d in healthy skin, psoriatic lesional and nonlesional skin and in synchronized HaCaT cells. Immunohistochemistry was performed on tissue samples and on cultured HaCaT cells using CD1d NOR 3.2 and CD1d51 monoclonal antibodies (mAb). In healthy and uninvolved psoriatic skin two distinct staining patterns were obtained depending on the antibodies used: CD1d was expressed by differentiated keratinocytes beneath the stratum corneum (CD1d NOR 3.2 mAb) or by the whole epidermis (CD1d51 mAb). In psoriatic lesions, both antibodies showed a strong and diffuse expression. To clarify the cause of these different staining patterns RT-PCR, real-time PCR and Western blot analysis were carried out on synchronized HaCaT cells. RT-PCR results showed that CD1d was not detectable in proliferating, nondifferentiated HaCaT cells, but CD1d expression appeared and increased as the cells started to differentiate. To a more precise quantification of CD1d gene expression, real-time PCR was performed with a different primer set. Interestingly, these results showed that CD1d was expressed most intensely by proliferating cells. Western blot analysis, using CD1d51 mAb, revealed two products, one at 30 kDa and one at 47 kDa. The expression of the 30 kDa form gradually increased with differentiation, while the 47 kDa form was present in proliferating cells and was not detectable in fully differentiated, nonproliferating cells. Here we provide the first evidence that CD1d exists in two isoforms that are distinctively expressed in HaCaT keratinocytes in a proliferation-differentiation dependent manner. This could be the result of an alternative splicing of the CD1d gene.

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β-Carotene Interferes with Gene Regulation by UVA in Keratinocytes

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Objective: assess at the level of gene expression whether β-carotene (βC) can interfere with UVA effects in keratinocytes. Methods: HaCat cells, treated with 1.5 μM βC, were UVA irradiated, and subjected to Affymetrix GeneChip® hybridization. Results: 193 genes were found regulated by UVA. Of those, the UVA effect was reduced by βC for 44, enhanced for 69, and not changed for 80 genes. βC reduced UVA-induced MMP10 expression, indicating reduced extracellular matrix degradation. Chromatin assembly factor I (CAF I), an indicator of DNA damage and heavily induced by UVA, was also decreased by βC. βC repressed CENP-E and several cyclins, suggesting mitotic arrest. Dual-specificity phosphatases, which preferably inactivate ERKs over SAPK/JNK, were induced by βC, indicating a net increase of SAPK/JNK signalling. Also, βC enhanced the UVA-induced upregulation of phosphatidyl-inositol 3 kinase (PI3K). PI3K plays a major role in regulating Ca influx into cells. As βC increased the transcription of Ca channels, the Ca influx into βC/UVA-treated cells should have been greatly facilitated. Signalling by Ca and SAPK/JNK can induce apoptosis. This is consistent with the increased abundance of apoptosis-inducing genes, e. g. TR3 and GADD153. Apoptosis induction was confirmed in an assay for caspase 3 activity. Conclusions: βC interacted with UVA via several mechanisms, the dominating effect being induction of apoptosis. Apoptosis occurred at physiological βC concentrations and was p53-independent, as HaCat cells carry an inactivated p53 gene. This suggests that βC should aid killing UV-damaged, precancerous skin cells.

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Clonal Analysis of Epidermal Cells Derived from Small Human Skin Punch Biopsies

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Normal human epidermis consists of two types of proliferating keratinocytes: stem cells, which have high proliferative potential, and transit-amplifying cells, which are destined to undergo terminal differentiation after a few rounds of cell division. It is known that, *in vivo*, stem cells express higher levels of the β1-integrins than transit-amplifying cells. In this study, we sought to define these epidermal subpopulations on the basis of phenotype and clonogenic potential. For this purpose we used single cell suspensions from small human skin punch biopsies. These cell suspensions were sorted flow cytometrically into a β_1-integrin weakly positive (dim) and strongly positive (bright) subpopulation. Subsequently, the clonogenic potential of these subpopulations was compared in multiwell cell culture experiments. An image system was used to detect colonies and determine their growth characteristics. In five consecutive experiments, it turned out that cell size in the β_1-integrin bright subpopulation increased when colonies aged, whereas this was constant in the dim subpopulation. The total number of colonies formed was higher in the β1-integrin dim than in the bright subpopulation. The growth rate of colonies in the β_1-integrin dim subpopulation exceeded that of the β1-integrin bright subpopulation. A combination of flow cytometric analysis and sorting, cell culture and image analysis offers the opportunity to characterise epidermal subpopulations phenotypically and functionally. With this combination it was shown that cultured keratinocytes from β_1-integrin dim and bright subpopulations differed with respect to morphology and growth characteristics.

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Elevated Expression of EMMPRIN (CD147) and Membrane-Type Matrix Metalloproteinases in Chronic Wounds

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Matrix metalloproteinases (MMPs) contribute to matrix remodeling in venous leg ulcers. Extracellular matrix metalloproteinase inducer (EMMPRIN) has been reported to increase MMP expression, and membrane-type-1-MMP or MT1-MMP has been implicated to activate MMPs. The present study examined whether and to what degree EMMPRIN, MMP-2, MT1-MMP and MT2-MMP were expressed in venous leg ulcers as well as the association with MMP activity. By preparing biopsies from healthy skin and lesional tissue from venous leg ulcers EMMPRIN, MMP-2, MT1-MMP and MT2-MMP were analysed by using zymography and immunohistochemistry. Our investigations provide direct evidence of increased proteolytic activity of MMP-2 which could be proven in lesional skin in comparison to healthy controls by zymography. Immunoreactive staining displays intense staining for EMMPRIN, MMP-2, MT1-MMP and MT2-MMP in dermal structures of venous leg ulcers, whereas solely EMMPRIN and MMP-2 are expressed in perivascular regions. Our findings indicate that venous leg ulcers are characterized by elevated expression of EMMPRIN, MMP-2, MT1-MMP and MT2-MMP. The immunohistological findings of skin alterations reflects the dynamic process of activation of soluble and membrane-bound MMPs, which may be highly induced by EMMPRIN. These data suggest for the first time that membrane-bound MMPs may favor enhanced turnover of the extracelluar matrix und support unrestrained matrix metalloproteinase activity in venous leg ulcers.

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PPARβ Functions During Adult Murine Skin Regeneration

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Many nuclear hormone receptors have been implicated in skin maturation and development. Among them, the retinoic acid pathway is required for normal epidermis development, and ligands for the thyroid and the glucocorticoid receptors are known to accelerate the epidermal barrier maturation of rat skin. PPARα ligands were shown to accelerate epidermal development of fetal rat skin explants, suggesting that PPARs belong to the group of nuclear hormone receptors participating in the epidermal differentiation. PPARβ is absent from the adult interfollicular epidermis. Interestingly, its expression is strongly up-regulated in this tissue after a skin injury. Consistent with an implication of PPARβ, wound repair is delayed in PPARβ mutant mice, due to impaired adhesion/migration, cell death and proliferation of the mutant keratinocytes. We present here findings showing that PPARβ is an crucial effector regulating keratinocyte behaviour during the healing of a skin wound and is therefore an interesting therapeutic target.

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Novel Targets for Cancer Chemoprevention: Blockade of Hedgehog Signaling Inhibits Basal Cell Carcinomas (BCCs) in ptch+/− Knockout Heterozygous Mice

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BCCs induced by solar UVB are the most common form of human malignancy, affecting millions of patients worldwide each year. Mutations in sonic hedgehog (shh) signaling genes including patched (ptch), shh and smoothened (smo) activate transcription factors of the Gli family that regulate target genes ultimately leading to BCCs development. Ptch+/− knockout mice develop BCCs and trichoblastomas following chronic exposure to UVB radiation resulting in activation of the shh signaling pathway. In animals, shh signaling is critical for normal morphogenesis and cyclopamine, a specific inhibitor of shh, causes abnormal segmental development. In this study we evaluated the effect of orally administered cyclopamine on UVB-induced BCCs development in ptch+/− mice. Mice were irradiated with UVB (240 mJ per cm^2 three times a week) for 35 weeks at which time 50% of the animals had one or more BCCs. At this time point, UVB irradiation was stopped and the mice were divided into two groups of 30 animals each group having approximately equal numbers of tumors. Group-1 mice received vehicle whereas group-2 mice received 0.1 mg percentage cyclopamine (as cyclodextran complex) in drinking water and the number of tumors recorded weekly. Mice treated with cyclopamine developed 35% fewer tumors compared to vehicle-treated controls by week 45. In these mice we also assessed the effect of cyclopamine treatment on the induction of cell cycle regulatory proteins. UVB-irradiated nontumor bearing skin of these mice manifested high expression of cyclins D1, A2 and B1; however, in the cyclopamine-treated group there was substantial reduction in the expression of these proteins. These results indicate that inhibitors of shh signaling block UVB-induced BCCs development in ptch+/− heterozygous knockout mice and suggest that this could provide a novel target for cancer chemoprevention.

219 [Oral 003]

Topical Tacrolimus Accelerates Tumor Formation and Carcinogenesis in Mouse Skin

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Tacrolimus is a potent, fungus macrolide immunosuppressant, widely used for liver and kidney transplantation. Recently, topical tacrolimus has been found to be effective treatment for atopic dermatitis (AD) because of the well known effect of T cell immunosuppression. Since 7% of patients undergoing systemic administration of tacrolimus for liver transplantation subsequently developed malignancy (Transplantation, 66, 1193, 1998), we investigated the effect of topical tacrolimus on skin carcinogenesis in one hundred and four mice. The dorsal skin of 7 w old female CD^{-1} mice was treated with the tumor initiator of 7, 12-dimethylbenz [α] anthracene (DMBA) at a dose of 0.2 μmol or acetone alone on day 1 of the experiment, followed by promoting treatment with 5 μg of 12-O-tetradecanoylphorbol-13-acetate (TPA), with or without 5 nmoles of tacrolimus, or neutral vehicle (control), twice a week for 20 weeks. Result revealed that after 14 week, there was marked synergy between tacrolimus and the DMBA/TPA regimen, with 0.47 ± 0.13 new tumors per mouse per week in the tacrolimus/DMBA/TPA group vs. 0.10 ± 0.025 in the DMBA/TPA group ($p < 0.01$). 8.0% of the tumors were squamous cell carcinomas and others were papillomas. In mice treated with DMBA plus tacrolimus alone without TPA, two carcinomas and two papillomas out of 26 mice were found. This dramatic synergy between topical tacrolimus and conventional carcinogens raises the spectre of a significant risk of skin carcinogenesis in patients with AD undergoing prolonged treatment with tacrolimus. Although it has been reported that cyclosporin A (CyA) or tacrolimus does not interfere with NK cells, patients with a low CD4/CD8 ratio were still reported during systemic administration of CyA, an immunosuppressant similar to tacrolimus (Cancer, 80, 1141, 1997). In this connection, it is not unlikely that tacrolimus potently suppresses NK cells. Great caution and restraint in prescribing this regimen seem advised, as does careful surveillance of skin lesions in patients treated with tacrolimus for prolonged periods.

220 [Oral 040]

Evidence for Effects of Melanocortin 1 Receptor Variants on Melanoma Cell Behaviour

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The melanocortin 1 receptor (MC1R) is a seven transmembrane G-protein coupled receptor which is expressed by melanocytes and melanoma cells. *MC1R* variants are causally associated with red hair and fair skin, and increase susceptibility to melanoma and nonmelanoma skin cancer. The increased risk of skin cancer could be secondary to *MC1R* variant-induced alterations in skin pigmentation. However, the results of case control studies, where skin type and hair colour have been included in the analysis, have suggested that some of this higher risk may arise via effects on pathways unrelated to pigmentation. In this study, we investigated the nonpigmentary effects of *MC1R* variants on melanoma cell behaviour using Mc1r-null B16G4F cells stably transfected with wild type and variant (Arg151Cys, Arg160Trp and Asp294His) human *MC1R*. The transfected lines are amelanotic, and allow for alterations in cell behaviour to be examined independently of the effects of *MC1R* variants on pigmentation. In growth inhibition studies, 10^{-9}–10^{-6} M αMSH caused a significant reduction in proliferation of wild type transfected B16G4F cells (n = 4 lines, $p < 0.001$), but did not suppress growth of any of the variant *MC1R* transfectants (n = 5 lines). Adhesion to fibronectin by the wild type *MC1R* transfectants was significantly decreased by 10^{-9}–10^{-6} M αMSH in each of the four separate lines ($p < 0.001$), but no alterations in binding to fibronectin were detected in the variant *MC1R* transfectants. These results show that human *MC1R* variants affect melanoma cell behaviour *In vitro*, raising the possibility that *MC1R* variants alter melanoma susceptibility and/or progression through effects on these pathways.

221 [Oral 043]

$Ptch^{+/-}$ Heterozygous Knockout Mice Over-Expressing Ornithine Decarboxylase: Possible Model for Multiple Basal Cell Carcinomas (BCCs) in Gorlin's Syndrome

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Multiple BCCs are a major feature in patients with Gorlin's Syndrome or the Nevoid Basal Cell Carcinoma Syndrome (NBCCS). Based upon discoveries in NBCCS, mutations in sonic hedgehog (shh) signaling genes including patched (ptch) are known to underlie BCCs development. $ptch^{+/-}$ knockout mice develop BCCs and trichoblastomas after chronic exposure to solar ultraviolet B (UVB) radiation. Because UVB is a potent inducer of cutaneous ornithine decarboxylase (ODC) activity, which drives the continued proliferation and clonal expansion of initiated cells leading to tumor development, we reasoned that over-expressing ODC in $ptch^{+/-}$ heterozygous mice might provide a model of enhanced BCCs development. We have generated $ptch^{+/-}$ mice over-expressing the ODC transgene, hereafter referred to as $ptch^{+/-}$/ODC TgN. Untreated skin at the age of seven months appeared grossly normal; however, histologic examination showed small basaloid lesions. Immunohistochemical studies of these tumor-like growths showed positive staining for β-galactosidase (β-gal), gli-1 and shh. Neither the wild-type nor their $ptch^{+/-}$ littermates manifest such lesions or positive staining for β-gal. Our parent $ptch^{+/-}$ are heterozygous for deletion of exon 1 and 2 and insertion of *lacZ* and *neo* genes. BCCs in $ptch^{+/-}$/ODC TgN mice lead to de-repressed transcription of the *lacZ* gene inserted in the inactivated ptch locus. β-gal staining demonstrates activation of shh signaling in skin/BCCs-like lesions in $ptch^{+/-}$/ODC TgN mice. Chronic UVB irradiation (180mJ per cm^2 twice a week) for 10 weeks resulted in multiple small visible tumors, which increased both in number and size after another 20 weeks of irradiation. Tumor incidence was 100% by week 20. Histologically, these lesions resemble human BCCs and trichoblastomas and show strong positive staining for β-gal, gli-1 and shh. Our data show that β-gal positive staining lesions also stain for gli-1/shh. These results indicate that $ptch^{+/-}$/ODC TgN mice may be a model for multiple BCCs in patients with NBCCS.

222 [Oral 044]

Successive Roles of UVB in Skin Carcinogenesis: p53 Mutations, Colonization, and Breaking the Stem Cell Barrier

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Upon acquiring a mutation, a single cell must expand into a clone before becoming significant for carcinogenesis. Forces driving clonal expansion, and the obstacles that must be overcome, are poorly understood. In a genetic mechanism, acquiring a second mutation conferring a proliferative advantage would enable the cell to expand autonomously. If carcinogen exposure instead induced a physiological change, clonal expansion would require the carcinogen's continued presence. To address this question, we studied microscopic *p53* immunopositive clones of keratinocytes in epidermal sheets of mouse skin. To control the carcinogen exposure, we irradiated mice with UVB for various times and observed the expansion of p53-mutated cells into clones in mouse epidermis. *p53*-mutant clones only grew during chronic UVB exposure and regressed after the cessation of UVB. Therefore, clonal expansion was not triggered by a proliferative mutation, but was instead continually driven by UVB. Unexpectedly, the clone size distribution showed periodicity with maxima at estimated intervals of 16 ± 6 cells, the size of the epidermal proliferating unit in murine dorsal skin. In the absence of UVB, rare "imprisoned clones" increased in cell number without increasing in area. We conclude that UV acts on three levels of carcinogenesis: (1) by causing p53 mutations (2) driving clonal expansion of such p53-mutated cells and (3) allowing mutated cells to break into an adjacent stem cell compartment, a physical barrier to clonal expansion. Colonizing adjacent stem cell compartments is the rate-limiting step in clonal expansion; sustained UVB enables the *p53*-mutant keratinocyte to colonize without incurring an additional mutation.

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A Novel Connexin26 Mutation in a Patient Diagnosed with Keratitis–Ichthyosis–Deafness Syndrome

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Keratitis-ichthyosis-deafness (KID) syndrome is a rare disorder characterized by erythrokeratoderma, deafness and keratitis. Scarring alopecia and squamous cell carcinoma can also occur. Most cases described so far were sporadic. We present evidence that KID syndrome can be caused by a novel mutation in the connexin26 (CX26) gene. This finding expands the spectrum of disorders caused by defects in connexin26 and implies the gene in normal corneal function, hair growth and carcinogenesis. We ascertained a patient suffering from KID syndrome. She is the only affected person in the family. She suffers from severe, extensive spiky erythrokeratoderma, bilateral sensory deafness and a vascularizing keratitis. She also had a squamous cell carcinoma removed at age 38. Small, misshapen teeth and hypotrichosis were also part of the phenotype. In CX26 the patient had a heterozygous GAC to AAC change in codon 50. This changes a conserved aspartic acid into an asparagine in the first extracellular domain (D50N). Because the G to A change abolishes an *Asp*I restriction site, we examined controls and the family by restriction analysis. The mutation was not present in 164 control alleles and could not be demonstrated in the mother and four sibs either. The D50N change is expected to influence local protein structure and probably affects voltage gating and channel assembly. There is some evidence that suggests there exists an interaction between connexin26 and the cytoskeleton. If present, this interaction may explain why only KID syndrome is associated with squamous cell carcinoma whereas other disorders caused by mutations in the connexin26 gene are not. Our findings are the first to establish connexin26 as a possible tumor suppressor gene in humans. Connexins were known to have this function in animals but not in humans. Further studies are needed to examine how connexin26 is tied into growth regulation and cell differentiation.

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Genome-Wide CpG Island Methylation and Promotor Methylation Analyses in Malignant Tumors of the Skin

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Epigenetic regulation of gene expression may play an important role in carcinogenesis of the skin. Genome DNA methylation is one of the most important mechanisms in epigenetic regulation. With the help of newly developped method, nonisotopic cytosine extension assay (in press) we analyzed CpG island-specific and non-CpG island-specific global genome methylation in various malignant tumors of the skin. In malignant melanoma, non-CpG island hypomethylation was observed, but CpG island methylation was not affected. Bisulfite treatment of the genome and following PCR and/or direct sequence revealed lack of p16 gene promotor methylation. The alteration of genome DNA methylation may be associated with the development of malignant melanoma. Its detailed mechanism is remained to be elucidated.

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Expression of Tight Junction-Associated Proteins (Occludin, ZO-1, Claudin-1, Claudin-4) in Squamous Cell Carcinoma and Bowen's Disease

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The epidermis, which is a typical stratified epithelia, has tight junctions (TJs) in the granular layer, as simple epithelia do. So far, abnormalities of TJs in tumors of simple epithelia have been reported. Thus, expressions of TJ-associated proteins (occludin, ZO-1, claudin-1 and claudin-4) were examined in normal human epidermis, five cases of squamous cell carcinoma and Bowen's disease, by immunofluorescence staining. In normal epidermis, occludin, ZO-1 and claudin-4 were expressed in the granular layer specifically or dominantly, whereas claudin-1 was expressed in the whole layers. In squamous cell carcinoma, occludin, ZO-1, claudin-1 and claudin-4 were expressed in keratinizing tumor cells such as cancer pearls. In tumor cells with poor differentiation, expression of occludin, ZO-1 and claudin-4 were decreased or absent in expression. On the other hands, expression of claudin-1 was increased in some, but decreased in other population of tumor cells with poor differentiation. In Bowen's disease, occludin, ZO-1, claudin-1 and claudin-4 were expressed in keratinizing cells. These findings showed that expression of these molecules are associated with keratinization in malignancy of keratinocytes, as well as normal epidermis, and that altered expression of claudin-1 in tumor cells with poor differentiation may be related with carcinogenesis of keratinocytes.

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Expression of Collagen XVII is Altered in Oral Precancers and Squamous Cell Carcinomas

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Collagen XVII (BP180) is a hemidesmosomal transmembrane component that may participate in signal transduction and keratinocyte motility, in addition to its role in keratinocyte adhesion to the basement membrane. In squamous cell carcinomas (SCCs), an up-regulated expression of several hemidesmosome components is observed. In this study, distribution of collagen XVII in oral precancers and SCCs was examined with immunohistochemical and *in situ* hybridization methods. Interestingly, collagen XVII was found to be down-regulated in the basal cells of oral dysplasias, whereas in the suprabasal cells of moderate and severe dysplasias collagen XVII expression was up-regulated, as well as in the central tumor cells of grade II and III carcinomas. Collagen XVII could be extracted *In vitro* from oral keratinocytes and tongue SCC cells, as well as from tongue SCC tissue, and an increased collagen XVII expression level was revealed in the malignant cell lines by immunoblotting. The effect of cytokines TGF-β1, TNF-α, EGF, IL-1 β, IL-6 and PMA in regulation of collagen XVII gene expression was studied in oral keratinocytes. Ribonuclease protection assay (RPA) showed a significant induction in collagen XVII mRNA synthesis only by PMA (phorbol myristate acetate). These results indicate that collagen XVII expression is abnormal in oral dysplasias and SCCs. The reduced expression in the basal cells of dysplastic mucosa is likely to reflect the disturbed adhesion to the underlying extracellular matrix. Collagen XVII up-regulation in suprabasal cells of dysplastic mucosa and in central carcinoma cells in the absence of hemidesmosome formation may reflect alternative function of collagen XVII besides its role in cell adhesion.

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Genetic Aberrations Addressed by Comparative Genomic Hybridization (CGH) in Keratoacanthomas (KAs); a Benign Lesion with Malignant Phenotype

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KAs are commonly occurring benign skin lesions localized to sun-exposed areas; there is an increased incidence of KAs among immunosuppressed patients. They typically develop rapidly and may show cellular atypia and infiltration like squamous cell carcinomas (SCC), but finally regress spontaneously. We have shown that KAs have a high degree of genetic instability as assessed by CGH, with 35.7% (25/70) of the lesions harbouring chromosomal abberrations. The same frequencies were found among immunosuppressed organ transplant recipients (36.4%, 20/55) and patients without immunosuppression (33.3%, 5/15), indicating a common pathogenetic pathway in both situations. A higher frequency were found in SCCs, where 70% (7/10) of lesions showed genetic aberrations. Recurrent aberrations, given as a fraction of lesions with aberrations, were gains on 8q (20.0%), 1p and 9q (each 16.0%), and deletions on 3p (20.0%), 9p (20.0%), 19p (20,0%) and 19q (16.0%). The total number of losses and gains were about the same (i.e. 47 vs. 52, respectively). Many of the most frequently occurring aberrations in KAs were not detected in any of the SCCs, whereas some aberrations were shared by both types of lesions. Aberrations were found in early as well as in late stage of tumor development, indicating a role for genetic instability in the progression as well as in involution of KAs. Aberrations were found among KAs without as well as with atypia and infiltrative growth, and there were no significant correlations between the degree of infiltration or atypia, respectively, and genetic aberrations, although there was a tendency for lesions with atypia to have aberrations. Thus, malignant phenotypic development does not appear to be driven by the detected genetic aberrations, that seems to be associated with the biological behaviour of KAs in general. More detailed studies of chromosomal areas with recurrent aberrations are needed for localization of putative genes that determine the biology of KAs, and that may distinguish them from SCCs.

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The Equilibrium Between Apoptosis Inhibiting and Promoting Genes in Cutaneous T Cell Lymphoma (CTCL)

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Since cutaneous T cell lymphoma lesions show only little mitotises, it is hypothesized that the increase of tumor mass is mainly due to the enhanced survival of malignant T cells. The survival of a cell depends on the equilibrium between apoptosis inhibitors and promoters. Here we investigate the expression of the apoptosis inhibitors bcl-xL and mcl-1 on the one side and the apoptosis promoters bcl-xs, bad and bax on the other side. The expression of apoptosis regulating proteins in CTCL cell lines was investigated by Western blotting. The expression and localization of apoptosis regulating proteins was determined by immunohistochemical stainings with antibodies directed against the proteins bcl-x, mcl-1, bad and bax. Inactivation of Bad by phosphorylation was studied by a phosphorylation-specific antibody. The function of bax was tested by the addition of the anticancer drug sulindac, which promotes cell death by bax induction. The Western blot experiments with CTCL cell lines show, that bcl-xL, mcl-1, bad and bax are present, but that the expression of the apoptosis promoter bcl-x is missing. Bad was partially inactivated by phosphorylation. Bax was also ineffective, as sulindac could not induce apoptosis in CTCL cell lines. The genes bcl-x, mcl-1, bad, and bax were also expressed in all tested CTCL skin lesions. In two patients we found a significant increase of mcl-1 expression in skin lesions during disease progression. Increased expression of Mcl-1 and synthesis of nonfunctional Bax and Bad disturb the equilibrium between apotosis promotors and inhibitors and may be responsible for enhanced survival and resistance of CTCL cells against sulindac.

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Agonists of Peroxisome Proliferator-Activated Receptor (PPAR) γ Inhibit Cell Growth in Malignant Melanoma

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Peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear receptor superfamily involved in adipocyte differentiation and glucose homeostasis. There is evidence that PPARγ may also act as a tumor suppressor. Here, we demonstrate expression of PPARγ in benign melanocytic naevi, different variants of primary cutaneous melanomas and melanoma metastases. PPARγ protein and PPARγ1 mRNA were also detected in human melanoma cell lines. The PPARγ-specific agonists 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, troglitazone, and rosiglitazone dose-dependently inhibited cell proliferation in four melanoma cell lines, while specific agonists of PPARα had no such effect. At a concentration of 50 μM rosiglitazone, the most potent PPARγ agonist tested, suppressed cell growth by approximately 90%. Apoptosis could be induced in melanoma cell lines by incubation with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In contrast, the growth inhibitory effect of PPARγ activation was independent of apoptosis and seemed to occur primarily through induction of cell-cycle arrest. Our data indicate that melanoma cell growth may be modulated through PPARγ.

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Spectral Karyotyping Demonstrates Genetically Unstable Skin-Homing T Lymphocytes in Cutaneous T Cell Lymphoma

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We established cell lines from skin biopsies from four patients (MF8, MF18, MF19, and MF31) in an early stage of cutaneous T cell lymphoma using growth medium containing IL-2 and IL-4. After 3 weeks of culture an outgrowth of the skin-homing T lymphocytes was obtained. Lymphocytes were analyzed using the molecular cytogenetic technique, spectral karyotyping (SKY) in order to detect hidden rearrangements. Two patients (MF18 and MF19) had normal karyotypes. Multiple nonclonal numerical aberrations were seen in MF8 and MF31. MF8 had two different T lymphocyte clones, one with trisomy of chromosome 21(12/20 cells), a second revealing loss of chromosome 22 (3/20 cells). This patient also had a clonal deletion, del(5)(p15.1), as well as several nonclonal aberrations. MF31 had a clonal deletion, del(17)(p12), and other nonclonal deletions involving chromosomes 2, 5, 10, 11, and two chromosomal rearrangements. MF18 was the only patient with two nonclonal balanced translocations (t(1;2)(q32;p21) and t(4; 10)(p15.2; q24). After two years, three of the patients had new skin biopsies taken from skin lesions from the same previous sites and new cell lines were established. SKY analysis revealed the presence of a T cell clone in MF8, with 4/20 cells containing trisomy 21; additionally a new clone (17/20 cells) was seen, with the del(18)(p11.2). MF31 had only one aberrant cell with del(17)(p12), all other cells were normal. MF18 revealed clonal deletions (ex. del(1)(p36.1) in 3/20 cells) and nonclonal deletions (chromosomes 4, 12, 17, and 18), and rearrangements involving chromosomes 3, 5, 6, 12, and 13. Thus, three of four patients had recurrent clonal and nonclonal deletions and structural aberrations, with one patient two years later, still exhibiting the same T lymphocytes clone (47,XX, + 21). Our findings demonstrate a very high degree of genetic instability among skin-homing T lymphocytes in patients with cutaneous T cell lymphoma, in early stages of the disease and thus confirms previous observations of cytogenetic analysis of blood lymphocytes of CTCL patients with advanced disease.

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Functional Characterization of Neurotensin Receptors in Human Cutaneous T Cell Lymphoma Malignant Lymphocytes

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Cutaneous T-cell lymphomas (CTCL) are a clonal proliferation of CD4+ T lymphocytes primarily involving the skin. Mycosis fungoides is an epidermotropic CD4 + CTCL, and a more aggressive form Sezary syndrome occurs when the malignant cells become nonepidermotropic. The role of neuropeptides on the growth and chemotaxis capacity of CTCL cells remains unknown. Here we report that CTCL cells, similarly to normal resting T lymphocytes, expressed neurotensin transcripts. We used an IL-2-dependent CTCL malignant T cell line derived from CTCL lesions in order to study the role of neurotensin on the proliferation and the migration of these malignant cells. First, we determined that these malignant cells expressed neurotensin receptor-1 (NT-1) but not neurotensin receptor-2 (NT-2) on their cell membrane. Functional results indicated that neurotensin did not stimulate the growth of the cell line. However, we found that it enhanced the spontaneous migration of the malignant cells as efficiently as other chemokines such fractalkine and stroma cell-derived factor-1 (SDF1). We demonstrated that in CTCL, the NT_1 receptor is responsible for NT-induced migration and that its engagement induces redistribution of the cytoplasmic tyrosine kinases focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK-2). This results suggests that neurotensin in skin can play a role in the disease by stimulating their chemotaxis capacity.

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Fine Mapping of 10q Deletions in Mycosis Fungoides

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Primary cutaneous T-cell lymphoma (CTCL) represents a heterogeneous group of extranodal non-Hodgkins' lymphomas of which mycosis fungoides is the most common. The pathogenesis of CTCL is poorly understood. Cytogenetic G-banding, fluorescence *in situ* hybridisation (FISH) and comparative genomic hybridisation (CGH) studies have all identified chromosomal abnormalities at 10q in mycosis fungoides/Sezary syndrome. Previous loss of heterozygosity (LOH) studies performed in our department using 8 microsatellite markers have shown two minimal areas of deletion at 10q23.3 and 10q24. Allelic loss was demonstrated in 10/44 patients (23%). This study fine maps the area of deletion on chromosome 10q using 20 microsatellite markers in the region 10q23.2–10q25.2. DNA extracted from patients' skin biopsies (45) and matched unaffected peripheral blood mononuclear cells (controls) was analysed for LOH at each of the microsatellite markers. Loss of heterozygosity is representative of an area of chromosomal loss in the tumour DNA compared with the normal control DNA from the same patient. In this study allelic loss was found in 15/45 samples (33%). Of these 15 patients: 1 had stage Ia disease, 1 had Ib disease, 12 had IIb disease and 1 had IVb disease. The frequency of allelic loss has increased from 23% to 33% using this extended panel of microsatellite markers between 10q23.2 and 10q25.2. The pattern of LOH confirms our previous findings of 2 minimal regions of deletion and further characterises these regions to lie at 10q23.3 (D10S185) and 10q24.2 (D10S221). This extensive allelotyping data serves to identify two common regions of deletion in mycosis fungoides which are potential sites of putative tumour suppressor genes involved in the pathogenesis of mycosis fungoides. These results also provide the basis for future candidate gene analysis within these 2 minimal regions of deletion.

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Chromosome 12q Breakpoint Associates with Cutaneous T-Cell Lymphoma

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Patients with cutaneous T-cell lymphoma (CTCL) show a large variety of chromosomal aberrations with no previously known common or specific aberration. Our aim was to seek the most common aberrations using *in situ* hybridization methods, that allow the identification of chromosomes taking part in translocations, and to further characterize them with locus-specific *in situ* hybridizations. Blood lymphocytes of seven consecutive patients with Sézary syndrome (SS) and four patients with mycosis fungoides (MF, stages IA to IIB) were cultured with standard methods and studied with 24-colour *in situ* hybridization (multicolour FISH, MFISH or spectral karyotyping, SKY). Chromosomal breakpoints of clonal cells were further defined with G-banding. The most common cytogenetic breakpoint was studied with 14 YAC-probes (CEPH, Jean Dausset, France) of a contig located in two main bands, with additional YACs depending on the translocation, and further defined with BACs (Research Genetics). Five SS-patients had clonal chromosome 12q aberrations, four of them with breakpoint in q21 or q22. The fifth aberration involved a short segment of 12q. Four MF patients showed clonal or nonclonal breakpoints in 12q, three of them in q21 or q22. The breakpoint in two SS-patients was specified with YAC-and BAC-probes to one band. Thus, defined break points are common in all stages of CTCL, and identification of candidate genes is in progress.

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Melanocortin 1 Receptor Gene Variants are Associated with an Increased Risk for Cutaneous Sporadic Melanoma in an Italian Population

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Melanocortin 1 receptor (MC1R) gene variants have been associated with phenotypical features such as fair skin and red hair as well as sun sensitivity which are known risk factors for melanoma. Recently, specific MC1R allelic variants have been also associated with an increased risk for the development of both familial and sporadic melanoma in Northern European and Australian populations. We investigated the relationship of MC1R allelic variants, phenotypic features and occurrence of sporadic melanoma in an Italian population, who has a darker skin type than the populations examined to date. A standardized personal interview, a total skin examination and molecular analysis of the entire coding sequence of the MC1R gene by DNA sequence analysis were performed in 100 patients with sporadic melanoma and 100 control subjects. Conditional logistic regression models were used to evaluate the association between allelic variants of the MC1R gene and risk of cutaneous sporadic melanoma. The odds ratio with a 95% confidence interval was used as association measure. Unreported MC1R allelic variants in addition to previously described variants have been detected in our study population. Analysis of all different MC1R gene variants combined showed that the presence of MC1R variants accounted for a higher melanoma risk which seemed independent of skin type and hair color and that the Val60Leu variant conferred the highest melanoma risk in our population.

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Genetic Alterations in Patients with Multiple Melanomas

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Germline mutations of the CDKN2A gene play a crucial role in the pathogenesis of familial melanoma: Although the occurrence of multiple primary melanomas may have a genetic background, the presence of a genetic predisposition in patients with multiple primary melanomas (MPM) in the absence of a family history has not yet been investigated in detail. Recent studies have demonstrated that allelic variants of the MC1R gene may increase the penetrance in patients with CDKN2A mutations. In order to identify a genetic susceptibility to the development of MPM, we analyzed the CDKN2A gene for germline and somatic mutations as well as MC1R gene for germline mutations in 14 patients with 2 or more primary melanomas and no evidence of a family history of the disease. Polymerase Chain Reaction and direct DNA sequencing were used to screen the CDKN2A and MC1R genes for germline mutations. Loss of heterozygosity (LOH) and microsatellite instability analysis at different loci on 9p21 (D9S974, D9S126, D9S171) were performed in a total of 30 tumor specimens of all MPM patients. Mutational analysis of genomic DNA showed germline mutations in exon 2 of the CDKN2A in 3 of 14 MPM patients and the presence of allelic variants of MC1R gene in 11 of 14 MPM patients. LOH at different microsatellite loci has been detected in 1 of 3 patients in 2 of 7 melanoma tissues confirming a double somatic mutation of the CDKN2A gene in 1 of 14 MPM patients. Based on our results, germline mutations of the CDKN2A gene associated with the presence of allelic variants of MC1R gene in patients with MPM but no family history, is highly suggestive of a genetic predisposition to the development of multiple melanomas. In addition, our data support the pathogenetic role of bi-allelic inactivation of the CDKN2A gene in melanoma development.

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Different Patterns of Chromosomal Aberrations in the Subtypes of Primary Cutaneous B-Cell lymphoma

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Introduction: Primary cutaneous B-cell lymphomas (PCBL) are a heterogeneous group of B-cell lymphomas primary manifesting in skin. They differ from their extracutaneous counterparts by a different clinical behavior and a different immunophenotype. In primary systemic B-cell neoplasms, the detection of recurrent chromosomal aberrations has contributed to lymphoma classification and has provided insights into the molecular mechanisms underlying lymphomagenesis. Surprisingly so far, with the exception of controversial PCR-based studies on the translocation t(14;18), there exist hardly any data on cytogenetic aberrations in PCBL. Methods: A collection of different subtypes of primary cutaneous B-cell lymphomas was analysed by comparative genomic hybridization (CGH) (n=23) and by fluorescence in-situ hybridization (FISH) (n=27). FISH probes flanking the *IGH, MLT, BCL6* and *MYC* gene loci were used. In case of breakpoints in these loci the translocation partners have been identified by translocation specific probes. Results: In 16/23 PCBL numerical aberrations have been detected by CGH. The more aggressive subtype of PCBL, the large B-cell lymphoma of the leg (LBCLL) shows more aberrations per case than the primary cutaneous follicle center cell lymphoma (PCFCCL). Gains in 18q and losses in 6q seem to be more frequent in LBCLL(8/13; 4/13) than in FCCL (1/10; 0/10). By FISH no translocations of the loci under study were detected in primary cutaneous marginal zone lymphomas (PCMZL) and in PCFCCL, but in 11/14 (79%) of LBCLL. Most frequently, the breakpoints affected the *IGH, MYC* or the *BCL6* locus. The most frequent translocation was t(8;14)(q24;q32) in 5/14 LBCLL. In further 5 cases there was a translocation involving the bcl-6 locus on 3q27, with translocation to the IgH locus (t(3;14)) in two cases and with translocation to the Ig-λ locus (t(3;22)) in one case. A translocation t(14;18) was not detected in any of the PCBL studied. Conclusion: There are strong cytogenetic differences between the subtypes of PCBL. Translocations with breakpoints in loci commonly involved in systemic B-cell lymphomas were detectable in 79% of LBCLL, but in none of the PCBL. LBCLL also shows more numerical aberrations, reflecting its more aggressive clinical behavior. In contrast to nodal B-cell lymphomas the translocation t(14;18) is of no significance in PCBL.

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Tumor–Stroma Interactions in Malignant Melanoma – Characterisation of a Novel cDNA

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In previous investigations we could demonstrate the activation of fibroblasts by tumour cells from malignant melanoma *In vitro*. The fibroblasts become activated by culture supernatants of melanoma cells, express elevated levels of MMPs and support the tumour cells to invade collagenous matrices *In vitro*. Beside the up-regulation of known genes, soluble mediators of melanoma cells are able to induce a so far unknown cDNA in fibroblasts detected by PCR-Select (Clontech) technology. Using RACE-PCR we have isolated the complete coding sequence giving a hypothetical protein of about 40 kDa. The cDNA is only weakly expressed in healthy skin and in biopsies of nonmalignant nevi detected by *in situ* hybridisation (ISH). The cDNA is strongly expressed in most of the melanomas (n = 15) investigated. However, there are different results on the expressing cells *in situ*: We have found melanomas with preferential expression by cells located in HMB45-positive regions suggesting that in contrast to *In vitro* data the melanoma cells themselves express this gene. In contrast, we have got also data on melanomas where the cDNA is expressed in regions staining for mAb AS02 (fibroblasts and activated endothelial cells) suggesting an activation of stromal cells known from *In vitro* experiments. The evaluation of clinical and ISH characteristics should give some answers whether differentiation of the tumour plays a role in the expression of this cDNA. Using *In vitro* expression studies we investigated the nature of the inducing agent (cytokine or chemokine?) in the supernatants of melanoma cells that induce the cDNA in fibroblasts *In vitro*. Fibroblast cultures have been exposed to various cytokines and chemokines in serum free medium and the gene expression has been analysed by RealTime RT-PCR. TGFβ1 and IL-1β obviously induce the expression of this mRNA in dermal fibroblasts *In vitro*. Investigations with blocking antibodies should show the significance of our results.

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Characterisation of the Cylindroma Tumour Suppressor Gene

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In this project, we investigate the molecular cause of adnexal skin tumour formation, in particular cylindroma and trichoepithelioma. In a large Swiss family affected with the Brooke–Spiegler syndrome (presence of cylindroma and trichoepithelioma), linkage analysis showed association to chromosome 16q21. Loss of heterozygosity analysis demonstrated that the same region was deleted in about 70% of the cylindroma tumour tissues arguing for the implication of this region in tumour formation. Mutational analysis in genes localized to the candidate interval revealed mutations in a single gene, named the cylindromatosis (CYLD) gene, in both familial and sporadic cylindromatosis cases. All mutations observed so far are either base substitution leading directly, or small deletion leading indirectly, to premature stop codons. Surprisingly, all mutations are clustered in the C-terminal two-thirds of the protein indicating possibly the presence of N-terminal domains exerting a dominant-negative effect of the truncated protein. The ubiquitously expressed CYLD gene encodes a protein of unknown function but contains several domains with homologies to CAP-Gly sites found in microtubule binding proteins, to the catalytic domain of deubiquitinating enzymes and to Zn finger proteins. Transfection of the full-length and mutant CYLD cDNA into different mammalian cells, as well as GFP-fusion proteins, demonstrated that wild-type and mutant proteins are localized to the cytoplasmic compartment. Coimmunoprecipitation experiments revealed the presence of several CYLD-associated proteins. Colocalization studies by confocal immunofluorescence microscopy, microtubule binding assays and the N-terminal sequence of the 50-KDa CYLD-associated protein suggest a weak association between the CYLD protein and β-tubulin. Yeast two-hybrid experiments using HaCat cell cDNA library as prey and full-length CYLD as bait, identified several CYLD interacting candidate proteins which are currently characterized further. These preliminary results suggest that CYLD is associated with microtubules during one part of the cell cycle and that its deubiquitinating activity could reduce the degradation of proteins suppressing cell proliferation or promoting apoptosis.

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Promoter Hypermethylation of the INK4A-ARF Locus in Skin Carcinomas

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The INK4A-ARF locus encodes two cell cycle-regulatory proteins, p16^{INK4a} and p14^{ARF}, that act through the Rb-CDK4 and p53 pathways. In our previous work, we have described UV-induced point mutations in this locus in 19% of squamous cell carcinomas (SCCs) and in 3,5% of basal cell carcinomas (BCCs). Considering that inactivation of INK4a-ARF locus by promoter hypermythylation is frequently detected in a large variety of tumors including melanoma and epithelial cancers, we have analyzed the promoter hypermethylation of the INK4A-ARF locus in 17 SCCs and 16 BCCs already analyzed for the presence of point mutation. Genomic DNA of skin tumors were treated by bisulfite reagent and amplified by Methylation Specific PCR (MSP) of the *p16* and *p14* gene promoters followed by direct sequencing of PCR product to verify the methylation status. We observed hypermethylation of the *p16* gene promoter in one out of 17 SCCs (6%) and in none of the BCCs. Surprisingly, we found hypermethylation of the *p14* gene promoter in four out of 17 SCCs (23,5%) and in 6 out of 16 BCCs (37,5%). Comparison of mutations and hypermethylation in the INK4a-ARF locus showed that only one BCC and one SCC presented simultaneously alteration and promoter hypermethylation of the *p14* gene while one other SCC had mutation and promoter hypermethylation of the *p16* gene. Our data demonstrate for the first time that the promoter hypermethylation of the INK4A-ARF locus is a frequent event in skin carcinomas, particularly in the *p14* gene promoter. Inactivation of the INK4a-ARF locus is a frequent event that occurs in 41% of SCCs and 37,5% of BCC, a prevalence closed to that described for the *p53* gene alteration (20% and 37%, respectively, in our tumors).

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Mechanisms of Inactivation of p16^{INK4a} & p14^{ARF} in Cutaneous Squamous Cell Carcinoma

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The CDKN2A locus on human chromosome 9p21 is implicated in the pathogenesis of cutaneous SCCs, but the precise mechanisms and genes involved have not been defined. CDKN2A encodes two candidate tumour suppressor genes (TSGs), p16^{INK4a} and p14^{ARF} which function as crucial cell cycle regulators in the Rb and p53 pathways, respectively. In addition to direct mutation, TSGs can be inactivated by promoter methylation, an epigenetic mechanism of gene silencing. The role of human papillomavirus (HPV) in the aetiology of cutaneous SCC remains controversial. If HPV plays a role, there may be genetic differences between HPV positive and HPV negative SCCs at key genetic loci, such as CDKN2A, where virus-derived proteins could abrogate TSG function by mechanisms other than mutation. We have investigated changes in p16^{INK4a} and p14^{ARF} in 40 cutaneous SCCs and correlated the results with tumour HPV status. Loss of Heterozygosity (LOH) studies, mutational analysis (using denaturing high performance liquid chromatography) and assessment of methylation status (with methylation specific PCR) were performed. LOH of 9p21 microsatellite markers was seen in 27% of informative cases. Solitary point mutations in exon 2 were discovered in 4/40 (10%) SCCs. These mutations create different single amino acid substitutions within p16^{INK4a} alone (n = 1), p14^{ARF} alone (n = 1) and both p16^{INK4a} and p14^{ARF} (n = 2). Promoter methylation of p16^{INK4a} and p14^{ARF} was detected in 11/28 (39%) and 13/31 (42%) cases, respectively. Three tumours had both a mutation and promoter methylation in the same gene: one SCC for p16^{INK4a} and two SCCs for p14^{ARF}. In all cases with biallelic events, an adverse effect on the function of the gene is likely with consequent loss of cell cycle control. There was no correlation between presence/absence of genetic/epigeneitc events and HPV status. This is the largest and most comprehensive study of the CDKN2A locus in cutaneous SCCs to date. Promoter methylation in cutaneous SCCs has never previously been demonstrated. For both p16^{INK4a} and p14^{ARF} promoter methylation seems to be a more important mechanism of gene inactivation than direct mutation in cutaneous SCC carcinogenesis.

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The Vitamin D System in Cutaneous Squamous Cell Carcinomas (SCC)

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We analyzed key components of the vitamin D system in cutaneous squamous cell carcinomas (SCC). Intensity of vitamin D receptor (VDR)-immunoreactivity was increased in SCCs as compared to normal human skin (HS). However, staining did not correlate with histological type or grading of skin tumors. Comparing VDR-staining with staining for Ki-67, cytokeratin 10, transglutaminase K and apoptotic cells (terminal UTP nucleotide end labeling assay), no correlation was found. In SCL-1 and SCL-2 squamous cell carcinoma cells, the majority of tumor cells revealed VDR-immunoreactivity *In vitro*. Incubation of SCl-1 and SCL-2 cells with $1,25(OH)_2D_3$ (10^{-8} M) resulted in a reduction of the number of Ki-67-positive tumor cells, indicating responsiveness of these cell lines to the antiproliferative effects of $1,25(OH)_2D_3$. Using real time PCR (LightCycler), we have quantified mRNA expression of vitamin D receptor (VDR) and of the major enzymes involved in the synthesis and metabolism of $1,25(OH)_2D_3$ (vitamin D-25-hydroxylase [25-OHase], 25-hydroxyvitamin D^{-1} α-hydroxylase [1α-OHase], 25-hydroxyvitamin D-24-hydroxylase [24-OHase]) in SCCs and HS. Expression levels were determined as ratios between target genes (VDR, 1α-OHase, 25-OHase, 24-OHase) and the reference gene GAPDH. Interestingly, ratios for VDR/GAPDH, 25-OHase/GAPDH, 1α-OHase/GAPDH and 24-OHase/GAPDH were significantly (Wilcoxon-Mann–Whitney-test) elevated in SCCs as compared to HS. Our findings indicate that (i) VDR protein as well as mRNA for VDR, 25-OHase, 1α-OHase and 24-OHase are increased in SCCs as compared to normal human skin. (ii) VDR expression in cutaneous SCCs is not exclusively regulated by the proliferative activity or by the differentiation of these tumor cells, but by additional, unknown mechanisms (iii) alterations in VDR expression and in the synthesis and metabolism of vitamin D metabolites may be involved in the growth regulation of SCCs (iv) SCCs may be considered as potential targets for therapy with new vitamin D analogs that exert little calcemic side-effects or by pharmacological modulation of $1,25(OH)_2D_3$ synthesis/metabolism in these tumor cells.etabolism in these tumor cells.

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L1 Adhesion Molecule in Human Primary Cutaneous Malignant Melanoma, Metastases of Malignant Melanoma and Acquired Melanocytic Nevi

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There is increasing evidence demonstrating an important function of the L1 adhesion molecule for tumorigenesis and tumor progression in various malignancies, including ovarian carcinoma. We have analyzed immunohistochemically L1 expression in paraffin embedded speciments of acquired melanocytic nevi (n = 26), primary cutaneous melanomas (n = 24), and cutaneous (n = 15) and lymph node (n = 9) metastases of malignant melanomas using highly specific mAb UJ127.11 upon antigen retrieval. We found an increase in L1 immunoreactivity in malignant melanomas and metastases of malignant melanomas as compared to acquired melanocytic nevi that was statistically significant ($p < 0.05$). Additionally, a correlation of L1 immunoreactivity with histological data of prognostic value such as Clark level was found. We have shown previously that CD171 is released in various cell types from the cell surface in a soluble form (L1-200) into the medium and and that a 32-kDa fragment (L1-32) is retained in the membrane. We have now detected soluble L1-200 by Western blotting in the conditioned medium of cultivated melanoma cells, with MelJuso giving the highest release. MelJuso cells also revealed the presence of L1-32. Recent data have shown that ADAM10 is involved in L1 shedding. ADAM10 was present in the lysate of all melanoma lines in the proform of 97 kDa. The proform has to be processed by pro-proteinconvertase to generate the active enzyme. Only MelJuso and col38 cells showed significant amounts of active ADAM10. In conclusion, our findings demonstrate that L1 is strongly expressed in primary cutaneous malignant melanoma and soluble L1-200 is released in the conditioned medium of cultivated melanoma cells, indicating that L1 ag may be of importance for progression and metastatic behaviour of malignant melanoma.

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Bcl-x_L Mediates Chemoresistance in Human Melanoma *In Vivo*

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Malignant melanoma is one of the most aggressive human neoplasms and responds poorly to numerous treatment modalities including chemotherapy. Expression of genes that regulate apoptotic cell death plays an important role in determining the sensitivity of tumor cells to chemotherapeutic intervention. Bcl-x_L is an antiapoptotic member of the Bcl-2 family and is universally expressed in human melanoma. It is reasonable to assume that high levels of Bcl-x_L are capable of blocking chemotherapy induced apoptosis thereby mediating chemoresistance. In this study we investigated the influence of Bcl-x_L overexpression in melanoma in a xenotransplantation SCID mouse model. A transfection system was used to examine the influence of Bcl-x_L expression in melanoma on chemotherapy treatment *in vivo*. Bcl-x_L overexpressing human melanoma tumors grown in SCID mice were significantly more chemoresistant to cisplatin and dacarbazine compared to the two controls (vector control, parental cell line). Treatment of Bcl-x_L overexpressing tumors with chemotherapy resulted in a significant decrease of apoptotic cells in comparison to the control groups. Based on these findings we suggest that Bcl-x_L is a chemoresistance factor in human melanoma and thereby may indirectly contribute to its chemoresistance and its poor prognosis.

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Analysis of Cutaneous T-Cell Lymphoma by Comparative Genomic Hybridisation (CGH): Correlation with Clinical Course

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Cutaneous T-Cell lymphomas (CTLC) are a rare group of malignancies with indolent to aggressive clinical course. Comparative genomic hybridization was used to analyse chromosomal imbalances (CI) in 31 patients with CTLC (19 patients with indolent forms and 12 patients with aggressive forms). Indolent forms included Mycosis fungoides and large cell anaplastic $CD30^+$-lymphoma and aggressive subtypes comprised Sézary Syndrome, cytotoxic $CD30^-$ lymphoma and additionally transformed disease subtypes. CI were seen in 23 patients (74%). Loss of chromosomal material was observed more often than gain (55% vs. 45%). Diminished chromatin (= loss) was localised most frequently (> 20%) to the chromosomal regions 6q, 10p/q, 13q and 17p. Enhancement of chromatin (= gain) occurred most frequently in the chromosomal regions 3q, 7p/q and 8p/q. Other chromosomal aberrations were seen with lower frequencies. The number of aberrations per patient sample varied between 0 and 19 and correlated with clinical tumor stages: from none in stage Ia to $9.43 \pm 3,56$ (mean ± S.E.M) in stage IVb. CI occured more frequently in the aggressive subtypes (7.92 ± 2.29) than in indolent (2.08 ± 0.6) subtypes. A high number of CI ($\geqslant 5$) was associated with shorter survival. Gain of chromatin in 7q and loss of chromatin in 13q correlated with a significantly shorter survival whereas the most frequently observed aberration (loss in 17p) did not influence the prognosis of the disease. In summary, CGH revealed recurring chromosomal gains and losses in CTLC. The association of the imbalances with the clinical course of the disease suggests that genes encoded at these loci influence tumor development and progression.

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Withdrawn

246 [Oral 014]

Absence of Type I Collagen Gene Transactivation Upon TGF-β Stimulation in $Fos^{-/-}$ Fibroblasts

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Fos and Jun proteins belong to the AP-1 family of transcription factors involved in type I collagen gene regulatory programs induced by various stimuli, including TGF-β. Investigating TGF-β responsiveness in a cellular context devoid of Fos expression, we identified that *COL1A2* transactivation previously identified as a TGF-β/Smad3 target, was not achieved by exogenous addition of TGF-β to $Fos^{-/-}$ mouse embryonic fibroblast cultures. A specific deficiency in Smad3-dependent signaling was identified, whereas Smad2-dependent gene transactivation and activation of ERK signaling in response to TGF-β was similar to that observed in wild-type fibroblasts. The defect in Smad3 signaling was specific for the *Fos* knockout, as it was not observed in either $JunB^{-/-}$ or $cJun^{-/-}$ fibroblasts. Ectopic transient expression of *Fos* or *Fra-1/Fosl1* into $Fos^{-/-}$ fibroblasts failed to restore COL1A2 gene expression in response to TGF-β, whereas $Fos^{fosl1/fosl1}$ fibroblasts exhibited functional Smad3 signaling. EMSA experiments demonstrated reduced Smad/DNA complex formation when comparing nuclear extracts from TGF-β-treated $Fos^{-/-}$ fibroblasts vs. those from wild-type cells. Transfection of a Smad3 expression vector, but not that of either TGF-β receptors or Smad4 alone, restored Smad3-dependent transcription in $Fos^{-/-}$ fibroblasts. Western and northern analyses revealed significantly reduced Smad3 expression in $Fos^{-/-}$ fibroblasts as compared to the wild-type cells, whereas identical levels of expression for Smad2, TGF-β receptors types I and II, Smad2,and inhibitory Smad7, were detected. Nuclear transfection of a Smad3 expression vector in $Fos^{-/-}$ fibroblasts restored Smad/DNA complex formation and up-regulation of endogenous COL1A2 gene expression by TGF-β. Our results demonstrate that Fos plays a critical role in allowing *Smad3* gene expression at levels sufficient to sustain COL1A2 gene transcription in response to TGF-β.

247 [Oral 042]

Tissue Inhibitor of Metalloproteinases-3 Induces Apoptosis in Melanoma Cells by Stabilization of Death Receptors

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Tissue inhibitors of metalloproteinases (TIMPs) are important regulators of matrix metalloproteinase (MMP) and adamalysin (ADAM) activity. We have previously shown that adenovirally expressed tissue inhibitor of metalloproteinases-3 (TIMP-3) induces apoptosis in melanoma cells and inhibits growth of human melanoma xenografts. Here, we studied the role of death receptors in apoptosis of A2058 melanoma cells induced by TIMP-3. Our results show, that exposure of cells to recombinant TIMP-3, N-terminal MMP inhibitory domain of TIMP-3, and to adenovirally expressed TIMP-3 results in stabilization of tumor necrosis factor receptor-1 (TNF-RI), FAS, and TNF-related apoptosis inducing ligand receptor-1 (TRAIL-RI) on A2058 cells and sensitizes them to apoptosis induced by their respective ligands. Stabilization of death receptors by TIMP-3 results in activation of caspase-8 and and caspase-3, and subsequent apoptosis is blocked by specific caspase-8 inhibitor (Z-IETD-FMK) and by pan-caspase inhibitor (Z-DEVD-FMK). Adenovirus-mediated expression of TIMP-3 in human melanoma xenografts in SCID mice resulted in increased immunostaining for TNF-RI, Fas, and cleaved caspase-3, and in apoptosis of melanoma cells. Taken together these results show that TIMP-3 promotes apoptosis in melanoma cells through stabilization of three distinct death receptors and activation of their apoptotic signaling cascade through caspase-8.

248 [Oral 048]

Roles of PPARs in Skin Wound Healing

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The α, β, and γ isotypes of the peroxisome proliferator-activated receptor (PPAR) belong to the nuclear hormone receptor superfamily. They are activated by fatty acids and eicosanoids, as well as by hypolipidemic and hypoglycemic drugs. With respect to the mouse epidermis, PPARs are expressed during foetal development but they disappear progressively from the interfollicular epithelium after birth. Interestingly, PPAR-α and -β expression is reactivated in the adult epidermis after various stimuli, resulting in keratinocyte proliferation and differentiation. Using PPAR-α, -β, and -γ mutant mice, we demonstrate that PPAR-α and -β are important for the rapid epithelialization of a skin wound and that each of them plays a specific role in this process. These findings reveal unpredicted roles for PPAR-α and -β in adult mouse epidermal repair. The immediate response to a skin injury is the release of inflammatory cytokines. Using primary cultures of murine keratinocytes from wild-type and PPAR-β −/− animals, we found that such signals, including TNF-α and IFN-γ induce keratinocyte differentiation. This cytokine-dependent cell differentiation pathway requires an up-regulation of the PPAR-β gene. Transcriptional stimulation of this gene occurs via the stress-associated kinase cascade, which targets an AP-1 site in the PPAR-β promoter. Interestingly, the pro-inflammatory cytokines also trigger the production of endogenous PPAR-β ligands, which are shown to be essential for activation of the receptor. We further show that activated PPAR-β regulates the expression of genes associated with the inhibition of apoptosis, which results in an increased resistance of keratinocytes to cell death after an injury and during wound healing.

249 [Oral 050]

1α,25(OH)$_2$D$_3$ Increases AP-1 DNA Binding Activity via the Annexin II Membrane Receptor by a PI3-Kinase/Ras/MEK/ERK1/2 and JNK1 Dependent Mechanism: *In Vitro* and *In Vivo* Regulation of AP-1 Binding Activity by 1α,25(OH)$_2$D$_3$ and Calcipotriol

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1α,25(OH)$_2$D$_3$ added to human keratinocytes increases differentiation through an activation of the transcription factor Activator Protein 1 (AP-1). The purpose of this study was to further investigate the mechanisms by which 1α,25(OH)$_2$D$_3$ and calcipotriol modulates AP-1 DNA binding activity *In vitro* and *in vivo*. Western blotting experiments revealed that 1α,25(OH)$_2$D$_3$ caused a rapid and transient activation of the MAP-kinases ERK1/2 and JNK1 in normal cultured human keratinocytes and also lead to an increased expression of the AP-1 subunits, c-Fos, Fra1 and c-Jun as determined by Northern and Western blotting. The 1α,25(OH)$_2$D$_3$-induced AP-1 DNA binding activity was completely blocked by the MEK/ERK inhibitor PD98059 indicating that the MEK/ERK pathway is involved in the activation of AP-1. Transfection experiments showed that 1α,25(OH)$_2$D$_3$ increases the AP-1-dependent transactivation, which was completely blocked by a dominant negative Ras. Preincubation of the keratinocytes with the specific PI3-kinase inhibitors, Wortmannin and LY294002, demonstrated that the 1α,25(OH)$_2$D$_3$-induced activation of ERK1/2 and JNK1 was mediated by a PI3-kinase dependent mechanism. Finally, preincubation of the keratinocytes with an antibody against the membrane receptor annexin II, blocked the 1α,25(OH)$_2$D$_3$-induced activation of ERK1/2 and JNK1. *In vivo*, AP-1 DNA binding activity was significantly reduced in keratome biopsies from involved psoriatic skin compared to un-involved psoriatic skin. The AP-1 subunits c-Fos, Fra1 and c-Jun were also reduced in psoriatic skin compared to un-involved psoriatic skin. However, topical application of calcipotriol to involved psoriatic skin for four days resulted in an increased AP-1 DNA binding activity when compared to vehicle. Our data show that 1α,25(OH)$_2$D$_3$, via binding to the membrane receptor annexin II, induces activation of the PI3-kinase/Ras/MEK/ERK1,2 and JNK1 signal transduction pathway resulting in increased expression of c-Fos, Fra1 and c-Jun and subsequently increased AP-1 DNA binding activity and gene transcription both *In vitro* and in involved psoriatic skin *in vivo*. Stimulation of AP-1 DNA binding activity via this signal transduction pathway may be important for the antipsoriatic effects of various vitamin D$_3$ analoques.

250 [Oral 051]

Cyclic Adenosine 3′,5′-Monophosphate Inhibits Transforming Growth Factor-β/Smad-Induced Type I Collagen Gene Transcriptional Activation via a PKA Dependent Mechanism

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Smad proteins transduce signals from TGF-β receptors to regulate transcription of target genes. To initiate specific gene transactivation, phosphorylated Smad3 associates with CBP (CREB-binding-protein) or closely related p300. Cyclic adenosine 3′,5′-monophosphate (cAMP) is the key second messenger in the cellular response to various hormones, neurotransmitters and neuropeptides. Among the latter a group of bioactive peptides, the melanocortins have been shown to regulate immune and inflammatory responses, hair growth, and extracellular matrix composition in human skin cells. Using either an artificial Smad3/4-dependent reporter construct or natural TGF-β responsive promoters, we show that synthetic cAMP prevents TGF-β-induced Smad-specific gene transactivation and type I collagen gene expression in a dose- and time-dependent manner. The level of intracellular cAMP is regulated by the balance between G$_s$ protein stimulated activity of adenylate cyclase (AC) and cAMP degrading cyclic nucleotide phosphodiesterase (PDE). In this context, we demonstrate that cAMP-induced inhibition of Smad signaling and collagen gene expression is mimicked by either AC stimulation or PDE inhibition. In addition, cAMP-induced repression of Smad-driven gene transactivation was completely abolished by preventing the activity of the main effector of increased intracellular cAMP levels, protein kinase A (PKA). Immunofluorescence and EMSA studies revealed that cAMP failed to inhibit nuclear translocation and DNA binding of Smad3/4 signaling complexes. On the other hand, using a mammalian two-hybrid system, we observed that Smad3–CBP/p300 interactions are abolished by forskolin. From these results, we provide a model of suppression of TGF-β/Smad signaling by cAMP-inducing agents, leading to reduced collagen gene expression: interference with Smad-driven transcription is achieved by PKA-dependent disruption of Smad complexes from transcriptional coactivators. Whether these mechanisms contribute to the role of neuropeptides such as α-MSH as regulators of fibrosis and wound healing remains to be elucidated.

251 [Oral 053]

Regulation of Stem Cell Factor Receptor Signaling by Cbl Family Proteins (Cbl-B/C-cbl)

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Cbl proteins, Cbl-b & C-cbl, function as ubiquitin ligases (E3) that have been implicated in the negative regulation of signaling of different nonreceptor and receptor tyrosine kinases. Whether the Stem Cell Factor (SCF) receptor, KIT, is regulated through the proteosomal pathway or the involvement of the Cbl-b/C-cbl in this regulation has not been elucidated. Through mammalian cell coexpression and coimmunoprecipitation studies, we show here that Cbl-b/C-cbl also acts as E3, binds and leads to the ubiquitination and down-regulation of KIT proteins, especially upon SCF stimulation. A proteasome inhibitor, lactacystin, blocked Cbl-b-mediated degradation of activated KIT. Intact tyrosine kinase-binding domain and the Ring finger domain of Cbl-b or C-cbl were essential for KIT degradation in 293T cells. Furthermore, the interaction between Cbl-b or C-cbl and KIT required the presence of activated Src kinases. Cbl-b or C-cbl mediated degradation of activated KIT was abolished by coexpression of a double mutant form of c-kit (c-kit$^{Y568F/Y570F}$), in which the docking sites for binding of Src family kinases in the juxtamembrane domain of c-kit were mutated. Inhibition of Src kinase activity with PP2, a specific Src kinase inhibitor, also blocked the degradation of activated KIT mediated by Cbl-b or C-cbl. Cbl-b, as well as C-cbl, was phosphorylated following activation of KIT. The phosphorylation of Cbl-b was primarily mediated by the Src family kinases. The c-kit$^{Y568F/Y570F}$ mutant blocked the phosphorylation of Cbl-b induced by SCF. In addition, we found that Cbl-b was degraded shortly after receptor engagement in mast cell lines, while prolonged treatment to mast cell lines with SCF led to the down-regulation of KIT and stabilization of Cbl-b. Taken together, our data suggest Cbl-b/C-cbl-mediated ubiquitination of KIT is essential for the degradation of activated SCF receptor, KIT.

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Mechanical Pressure Induces p38 Phosphorylation in Human Keratinocytes

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Human skin is permanently challenged by mechanical forces of different qualities. Mechanical stretching which is considered as a proliferation stimulus is presented, e.g. in abdominal skin enlargement during pregnancy. Vice versa other locations like the palms of the hand or the foot soles are more prone to mechanical pressure. In this context mechanical pressure is thought to trigger differentiation processes. Additionally, mechanical pressure is also discussed as a trigger factor of some skin diseases (Koebner phenomena). The underlying molecular signalling pathways which are involved in transduction of mechanical pressure are still enigmatic yet. In the present *In vitro* attempt HaCaT cells were mechanically stimulated by insertion of a Teflon weight into the culture dish (0.015 N per cm^2). After different time intervals total protein was extracted and analysed for activation of members of the mitogen-activated protein kinases (MAPK). Mechanical pressure applied for a total time of 30min showed a peak phosphorylation for p38 at 10min. The extracellular signal-regulated kinases ERK1/2 showed no alteration in the phosphorylation state. In order to further dissect the signalling cascade upstream and downstream regulators of p38 were examined. Mechanical pressure induced the phosphorylation of MKK3/6 indicating this kinase to be involved in p38 activation. Downstream from p38 the phosphorylation of the small heat shock protein HSP27 could be demonstrated. Among the well established stimulators of p38 are osmotic stress, heat shock, and inflammatory cytokines, which suggest p38 to be involved in the cellular stress response. Our data feature mechanical pressure as a new type of cellular stress which yields in p38 activation. As p38 plays an important role in the IL-8 induction we suggest mechanical pressure as a pro-inflammatory stimulus. These findings may contribute to the molecular understanding of mechanically triggered skin diseases like psoriasis and vitiligo.

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Granulocyte Macrophage-Colony-Stimulating Factor (GM-CSF) Treatment of Chronic Venous Ulcers Induces VEGF Up-Regulation

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Granulocyte macrophage-colony-stimulating factor (GM-CSF) has been successfully employed in the treatment of chronic cutaneous ulcers, but its mechanism of action has not been completely clarified. In this study, we investigated whether GM-CSF treatment of chronic ulcers promotes neovascularization of the granulation tissue and if this effect correlates with local induction of vascular endothelial growth factor (VEGF) and of the functionally related placenta growth factor (PlGF). Seven patients affected by chronic venous ulcers were treated with human recombinant (h) GM-CSF and skin biopsies were taken at the wound edge before (day 0) and five days after treatment (day 5). Computer-assisted morphometric analysis on tissue sections stained with an antibody recognising endothelial cells (anti-PECAM/CD31) or smooth muscle cells (antia-smooth muscle actin) revealed that small-sized vessel density in the granulation tissue was significantly increased after GM-CSF treatment. Specimens were then analysed for VEGF and PlGF expression by *in situ* hybridisation (ISH). After GM-CSF treatment, a strong up-regulation of the hybridisation signal for VEGF mRNA was detected in migrating keratinocytes at the wound edge and in cells within the granulation tissue. On the other hand, PlGF mRNA was faintly transcribed by migrating keratinocytes at day 0 and not significantly increased at day 5. Immunohistochemistry, performed with an anti-CD68 antibody on sections serial to those used for ISH, indicated that several of the cells actively transcribing VEGF within the granulation tissue were monocyte-macrophages. To analyse the molecular mechanisms underlying our *in vivo* observations, we carried out Northern blot and ELISA analysis on cultured human keratinocytes and on the U937 monocytic cell line treated with hrGM-CSF. No significant increase in VEGF, both at the RNA and protein level, was detected in GM-CSF-treated keratinocytes, while VEGF mRNA was up-regulated in the monocytic cell line. Taken together, our results indicate that GM-CSF treatment of chronic venous ulcers promotes neovascularization. This effect may be mediated by a direct induction of VEGF in monocyte-macrophage cells and an indirect VEGF induction in keratinocytes.

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Functional Specificity and Diversity of the Cyclin-Dependent Kinase Inhibitors $p27^{KIP1}$ and $p21^{CIP1}$ in T Cell Anergy – Towards New Models for Autoimmunity

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The molecular events governing the induction and maintanance of T cell anergy are poorly understood. Recent evidence points to a central role of basal cell cycle regulators, particularly the cyclin-dependent kinase (CDK) inhibitors, $p27^{KIP1}$ and $p21^{CIP1}$, in the regulation of anergy. Key evidences include that (i) $p27^{KIP1}$ protein levels are not down-regulated in anergic in contrast to productively stimulated T cells; (ii) overexpression of $p27^{KIP1}$ in T cells mimick an anergy-like G_1 arrest; (iii) the association of $p27^{KIP1}$ with the c-Jun coactivator JAB1 could be involved in the decreased transactivation of the *IL-2* gene; (iv) unexpectedly, $p21^{CIP1-/-}$ mice develop a lupus-like syndrom with age. However, the precise function of these CDK inhibitors in T cells has remained elusive. To rigorously evaluate the requirement of $p27^{KIP1}$ and $p21^{CIP1}$ for T cell anergy in comparison to other nonproliferative states, we analyze $p27^{KIP1-/-}$, $p21^{CIP1-/-}$, and corresponding wild-type mice in various scenarios. Phenotypically, purified $CD4^+$ $p27^{KIP1-/-}$ T cells show an increased fraction of $CD25^+$ cells before stimulation, a shortened cell cycle, and an accelerated response to stimulation, while $p21^{CIP1-/-}$ T cells closely resemble wild-type cells. In contrast, the induction and maintainance of proliferative quiescence is unaltered, indicating that neither $p27^{KIP1}$ nor $p21^{CIP1}$ are required for this response. Strikingly, $p27^{KIP1-/-}$ T cells exhibit partial resistance to anergy induction *In vitro* and *in vivo* as demonstrated by increased cell duplication, increased BrdU incorporation, CD25 up-regulation, synthesis of CDK4/cyclin D3 complexes, and retinoblastoma protein hyperphosphorylation. In comparison, $p21^{CIP1-/-}$ T cells become fully anergic as judged by these criteria, but show an augmented proliferative response following restimulation postanergy induction. In conclusion, $p27^{KIP1}$ and $p21^{CIP1}$ act as specific regulators of T cell anergy in overlapping but distinct pathways rather than general inhibitors of T cell proliferation. Since each regulator individually is not absolutely required for T cell anergy, current work aims to identify additional cell cycle regulators that function specifically in T cell anergy vs. quiescence.

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Transactivation of EGF-Receptors and Induction of c-fos Expression by Angiotensin II in Human Dermal Fibroblasts

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Angiotensin receptors are expressed by diverse human cutaneous cells including dermal fibroblasts. This study was designed to identify angiotensin receptor coupled second messengers in this cell type. Signal transduction mechanisms known to be coupled to angiotensin receptors on fibroblasts of other origin comprise the transactivation of EGF-receptors and, consecutively, the induction of c-fos expression. Primary dermal fibroblasts, isolated from human foreskin, were incubated for 1–5 min (for determination of EGF-receptor transactivation) or 15–60 min (for estimation of c-fos expression), respectively, with angiotensin II (10-7-10-11 M). Total RNA was isolated from treated or untreated (control) cells and forwarded to semiquantitative RT-PCR analysis of c-fos transcripts. The protein fractions of treated and untreated cells were immunoprecipitated using an antibody directed against the phosphorylated, activated EGF-receptor and subsequently analysed by Western Blotting. Angiotensin II time-dependently elicited a transactivation of EGF-receptors with a maximal effect as early as 2 min after stimulation. It, furthermore, time- and dose-dependently induced c-fos expression, peaking after 30 min at a concentration of 10–7 M angiotensin II. From our results we conclude, that human dermal fibroblasts express functional angiotensin receptors. Physiological functions of angiotensin II in fibroblasts derived from noncutaneous tissues, which are transmitted by a transactivation of EGF-receptors or by an induction of c-fos expression, include the stimulation of fibronectin- and TGF-β-synthesis. It is currently under investigation, whether physiological actions of angiotensin II in human skin may involve fibrosis.

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Vascular Endothelial Growth Factor-C Expression in Primary Cutaneous Melanomas Associates with Tumor-Positive Sentinel Lymph Node

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The presence of lymph node metastases is of crucial relevance for the prognosis of most human tumours, but little is known about the molecular mechanisms which guide metastasis spreading via the lymphatic system towards regional lymph nodes. Vascular endothelial growth factor-C (VEGF-C), a member of the vascular endothelial growth factor (VEGF) family, has been demonstrated to be involved in tumour-induced lymphangiogenesis and lymph node localization of metastases. In a previous study, we have shown that VEGF-C is expressed by the majority of lymph node melanoma metastases analysed, suggesting that the production of this growth factor by primary cutaneous melanomas may be associated with tumour spreading via the lymphatics. To further investigate this aspect, we have selected 18 cases of primary skin melanomas with metastases at sentinel lymph node biopsy, observed in our Institute between 1998 and 2000. For each case, a comparison patient with primary melanoma but negative sentinel lymph node biopsy, matched for age, sex and Breslow's thickness has been identified. The expression of VEGF-C on the 36 primary melanoma specimens has been evaluated by immunohistochemistry by two independent, blind observers. VEGF-C expression was detected in 89% of patients with tumor-positive sentinel lymph node vs. 22% of control patients. The observed difference in VEGF-C expression was found to be significant ($p = 0.000$, McNemar test). Our results show that VEGF-C production by the primary tumour is associated with the presence of lymph nodal metastases. In order to better understand the predictive role of VEGF-C expression in melanoma metastatisation we are analysing lymphangiogenesis in the primary lesions.

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Autocrine Ephrin-B2 – Eph Receptor Tyrosine Kinase Signaling Enhances Integrin-Mediated Attachment of Melanoma Cells to ECM Components

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Eph receptors are a unique family of receptor tyrosine kinases (TK) that play a pivotal role in embryonic patterning and neuronal development. This is partially attributable to modulation of cell migration and ECM-adhesion by cell-to-cell signaling. We have previously demonstrated that advanced melanomas (MM) constitutively overexpress both ephrin-B2, a cell surface bound ligand of this system, and the corresponding cell surface receptor-TKs of the B-class, suggesting autocrine stimulatory loops in MM. Currently, the functional consequences for MM progression are unknown. The aim of this study was to find out, whether cell attachment to ECM-components is altered by ephrin-B2-ephTK signalling. A panel of 20 MM-cell lines, mouse and human, was screened by real time PCR for relative expression levels of ephrin-B2. Surprisingly, mouse B16 MM-cells lacked ephrin-B2 expression completely, whereas most of the others showed varying expression levels. A full-length mouse ephrin-B2 expression construct was therefore designed and stably transfected B16-clones were established. Overexpression was confirmed by both FACS and Western blotting. Attachment assays were performed by seeding transfectants onto surfaces coated with laminin, fibronectin, and collagen. Ephrin-B2 overexpressing clones showed a significantly increased attachment to laminin (LM) and fibronectin (FN), but not collagen, suggesting the involvement of specific integrins. The number of attached cells after 30 min incubation was about doubled when compared with mock-transfected cells ($p < 0.05$). To further confirm the activation of RGD-binding integrins, RGD peptide (Gly-Arg-Gly-Asp-Ser) was used as a specific competitor. Consistently, the increase of attachment could be blocked completely with μM RGD-doses. To find out if signaling competence of ephrin-B2 cell surface bound ligand is required we knocked out the cytoplasmic signaling domain of ephrin-B2. B16 stably transfected with this truncated version did not show a significant increase of ECM-attachment. Taken together, we collected evidence that autocrine bidirectional ephrin-B2-ephTK signaling among MM-cells could be an important mechanism of activation of FN- and LM-binding integrins, which determine MM-cell adhesion, spread, and possibly metastatic growth.

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Smad7 Represses PAI-1 Promoter Activity in Human Dermal Endothelial Cells

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Smad proteins are intracellular proteins which mediate the TGF-β superfamily signaling from the activated TGF-β receptor complex to the nucleus. Ligand-specific receptor-associated Smads bind to the actived receptor type I and consist of Smad1, 5, 8 for the BMP-signaling cascade and of Smad2, 3 mediating TGF-β signaling. Once bound to co-Smad Smad4, they translocate into the nucleus where they bind specific DNA targets either alone or in association with other transcription factors. Inhibitory Smads, Smad6 and Smad7, block TGF-β superfamily signaling by binding activated type I receptors, thereby preventing receptor-associated Smad phosphorylation and subsequent nuclear translocation. Smad7 expression is up-regulated by laminar shear stress in endothelial cells. Until now, although Smad7 can be found in the cell nucleus, little is known about its nuclear role. Plasminogen activator inhibitor-1 (PAI-1) plays a major role in the control of physiologically important mechanisms involved in the homeostasis of blood coagulation and remodeling of extracellular matrix. Using transient cell transfections of endothelial cells we demonstrate that Smad7 represses the activity of a PAI-1 minimal promoter in a dose-dependent manner. To get insights into the molecular mechanisms underlying Smad7 effect, we used fusion proteins of Smad7 fused to the Gal-4 DNA binding domain together with core promoter-specific reporter constructs. These core promoters contain either a cannonical TATA box or a combination of TATA box with an initiator site (INR) or just an initiator site. The initiator site is a pyramidine rich region which facilitates initiation of promoter transcription that can be found either alone or in combination with a TATA box. We demonstrate that Smad7 represses TATA box- containing as well as TATA-INR-containing core promoters. Repression does not depend on the phosphorylation state of Smad7 since a phophorylation deficient Smad7–249 A still represses in a similar extent. In opposite, TATA-less, INR dependent core promoters do not become repressed. These results suggest that Smad7 interacts either directly with the basal transcription machinery or via a transcriptional coactivator like p300/CBP, one of the best characterized transcriptional coactivators. When using a mammalian two hybrid assay in endothelial cells however, Smad7 and p300 did not interact with each other. The exact mechanisms of gene repression by Smad7 in endothelial cells is currently investigated.

259 [Oral 010]

Vaccination of Cutaneous T-Cell Lymphoma Patients with Tumor Lysate Pulsed Dendritic Cells

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Vaccination with autologous dendritic cells (DC) has been shown to be a promising approach in the treatment of malignant melanoma. With the use of tumor lysate as a source of tumor antigen other malignant cutaneous diseases such as cutaneous lymphoma become accessible for vaccination therapy. We analyzed 10 patients with cutaneous T-cell lymphoma (CTCL) (stage I-IV), who were treated with tumor lysate pulsed dendritic cells from 1999 to 2001 on an outpatient basis in our hospital. The preparation of tumor lysate included 4 freeze/thaw cycles. DC were pulsed with 100ig per ml tumor lysate protein equivalent and keyhole limpet hemocyanin (50 μg per ml). Thereafter, injection of 1×10^6 DC into noninvolved lymph nodes was performed once weekly. Out of 10 treated patients 4 patients (40%) presented with an objective response. Three patients presented with a partial response meaning a > 50% decrease of lesions lasting at least for one month. One patient showed a complete response. On average 6.6 ± 1.4 vaccinations were necessary for the induction of a clinical response. Interestingly, in contrast to our experience in melanoma patients, relapse occured within 2–4 weeks after treatment stop. Continuation of vaccination with new tumor lysate of progressive lesions in 2 patients induced again a treatment response. In all patients treated, vaccination therapy was well tolerated. Immunohistology showed massive infiltration of CD8 and TIA positive cells at the site of regressing lesions during treatment as well as molecular remission as assessed by T cell receptor γ chain polymerase chain reaction density gradient electrophoresis. Eight patients presented with a mild to strong DTH skin reaction toward tumor lysate after DC vaccination with heavy infiltrates of CD4+, CD8+ and CD45RO+ lymphocytes. We conclude that vaccination of CTCL using tumor lysate pulsed DC is well-tolerated and achieves clinical regressions in some patients. Compared to the vaccination in melanoma patients this strategy facilitates the close monitoring of the obvious clinical course during vaccination treatment.

260 [Oral 034]

Decreased Serum IgG Levels and Circulating B Cell Numbers are Responsible for Development of Anticonvulsant Hypersensitivity Syndrome Through Reactivation of HHV-6

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Anticonvulsant hypersensitivity syndrome (AHS) is a life-threatening drug-induced reaction. Although reactivation of human herpesvirus 6 (HHV-6) has recently been shown to contribute to the development of AHS, it remains unknown why prolonged exposure to certain drugs could result in HHV-6 reactivation. We therefore investigated whether immune dysfunction that may allow HHV-6 reactivation could be specifically found in patients with AHS. In this study, various immunological parameters including serum Ig levels and T/B cell counts were studied at various time points in 10 patients with AHS. Two groups of controls were enrolled: one group was those who received anticonvulsants for > 3 months without any rashes; and the other was those who developed maculopapular rashes without systemic involvement. The serum IgG levels (mean ± SEM, 723 ± 157) were dramatically decreased at initial evaluation in patients with AHS as compared with the two groups of controls (1558 ± 294; 1210 ± 219): in particular, IgG1 and IgG3 levels were decreased. Circulating B cells were also decreased in number (95 ± 55 vs. 247 ± 131) in patients with AHS. These alterations clearly preceded the increase in serum HHV-6 DNA and anti-HHV-6 titers, which were usually detected 2–4 weeks after onset of the clinical symptoms. Upon resolution of the clinical symptoms, these alterations were restored. The temporal relationship between development of AHS and the decrease in serum IgG levels and circulating B cells provides strong evidence of a causal relation between reactivation of HHV-6 and development of AHS. Our findings also suggest the clinical utility of monitoring serum IgG levels and circulating B cell numbers for predicting the onset of AHS.

261 [Oral 035]

Treatment of Toxic Epidermal Necrolysis with High-Dose Intravenous Immunoglobulin: Multicentre Retrospective Analysis of 48 Consecutive Cases

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Toxic epidermal necrolysis (TEN, Lyell's syndrome) is a rare adverse cutaneous drug reaction characterized by an average mortality of 30%. No specific treatment exists to date. TEN is caused by extensive Fas-mediated keratinocyte death, a process that can be inhibited by high dose intravenous immunoglobulin (IVIG) *In vitro*. Here we have evaluated the therapeutic potential of IVIG in TEN and parameters that may affect response to treatment. Forty-eight consecutive patients with TEN (> 10% body surface area skin detachment, mean 44.8%, range 10–95%) were treated with standard care and IVIG (mean total dose 2.7 g per kg, range 0.8–5.8 g per kg) in 14 dermatology centers. Objective response to treatment, final outcome at day 45, and parameters that may affect response to treatment were retrospectively analyzed. Analysis of the Fas-inhibitory activity of different IVIG batches was also performed. IVIG infusion was associated with a rapid (mean of 2.3 d, range 1–6 d) interruption of skin and mucosal detachment in 89.6% of patients, and 87.5% overall survival. Patients that responded to IVIG had received on average higher doses of IVIG and earlier treatment. Furthermore, analysis of 35 IVIG batches revealed important batch to batch variations in their capacity to inhibit Fas-mediated cell death *In vitro*. Early infusion of high dose intravenous immunoglobulin is safe, well tolerated and likely to be effective in improving the survival of patients with TEN. From our data we would recommend early treatment with IVIG at a total dose of 3 g per kg over 3 consecutive days (1 g per kg per day). The Fas-inhibitory activity of IVIG batches available for therapeutic use and tested herein varies from batch to batch.

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Development and Characterization of Optimized Hapten-Carrier Conjugates for Improved Diagnosis of Immediate Chemical Allergy

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At present the diagnosis of IgE-mediated hypersensitivity to chemicals such as phthalic anhydride (PA) is based on conjugates that are not characterized or standardized. The aim of this study was to develop optimized and molecularly characterized PA-conjugates that can be used to improve the diagnosis of PA-allergy. The PA-conjugates were synthesized and the number of haptens bound on a carrier protein was estimated by matrix-assisted laser desorption/ionization time of light (MALDI-TOF) mass spectrometry. Conjugates ability to bind IgE antibodies was measured by the enzyme-linked immunosorbent assay (ELISA). *In vivo* reactivity of the conjugates was evaluated by skin prick testing. The most active IgE binding conjugates had a PA:HSA molar ratio of 80. In the optimal conjugates the average numbers of PA haptens per HSA carrier molecule were 14–16. In ELISA, all 13 patients and none of the 20 controls had IgE antibodies to optimized PA-conjugate. The sensitivity and specificity of the ELISA was comparable or even better to commercial CAP RAST. PA-conjugates elicited positive test results in skin prick testing showing that conjugates are immunologically active also *in vivo*. These results indicate that optimized and molecularly characterized PA-HSA conjugates can be used both *In vitro* and *in vivo* assays to improve the diagnosis of PA allergy. Similar approach may be useful also with other chemicals capable of inducing immediate allergy.

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Level of Total Fibronectin and its Functionally High-Grade Fraction in Plasma of Patients with Psoriasis

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Methods: the level of total fibronectin (FN) in plasma of 89 patients with psoriasis was investigated. Besides total FN, at the surveyed patients simultaneous determination of its fraction capable at presence heparin on a cold to form precipitate, consisting of FN and fibrinogen was carried out. The formation of such precipitate is possible only at presence of the free and intact sites for linkage of appropriate ligands (heparin and fibrinogen) in FN molecule and consequently it in the certain measure characterizes functional full value of FN. 49 healthy volunteers made up control group. Results: it was found, that the level of total FN in plasma of patients with psoriasis is significantly higher in comparison with control group (accordingly 398 ± 11.7 mcg per ml and 331.5 ± 11.1 mcg per ml; $p < 0.01$). Thus, significant decrease of the functionally high-grade FN maintenance (191.4 ± 8.1 mcg per ml was marked at norm 255.6 ± 17.1 mcg per ml; $p < 0.01$). Probably, this decrease could be the consequence of FN linkages in bloodstream with excess of circulating ligands, and proteolytic damages of FN molecule.

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Peripheral Blood Cellular Responses to Tetanus Toxoid and Tuberculin are Suppressed After Long-Term Topical Glucocorticosteroid Usage

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Topical corticosteroids do permeate skin and prolonged systemic corticosteroid use also causes systemic immune suppression. We wanted to study the effect of long-term topical corticosteroid use on peripheral blood immune responses to bacterial antigens and to nonspecific stimulation by PHA. Age-matched groups of patients were studied, i.e. 15 healthy volunteers with no history of topical corticosteroid use, and 16 patients who had been using topical corticosteroids for several years. The response of peripheral blood mononuclear cells (PBMC) to tetanus toxoid and tuberculin were measured by H3-thymidine incorporation, as well as IgE, IgG1, IgG2, IgG3 and IgG4 levels to same antigens by in-house ELISA's. Humoral immune responses, as measured with ELISA, were not altered by long-term topical corticosteroid use. However, proliferative responses of PBMC were significantly suppressed to tetanus toxoid ($p < 0.05$), tuberculin antigen ($p < 0.05$) and to nonspecific PHA stimulation ($p < 0.05$) in the patient group that had been using topical corticosteroids for prolonged periods. It seems that peripheral blood cellular immune responses are indeed suppressed after long-term topical corticosteroid use, possibly due to percutaneous absorption.

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47 Patients in 14 Families with the Rare Genodermatosis Keratosis Punctata Palmoplantaris Buschke-Fischer-Brauer

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We summarize the clinical data of 47 patients with the rare genodermatosis keratosis punctata palmoplantaris Buschke-Fischer-Brauer. The pedigrees of 14 German families were studied. In three families there was only one member affected, two or more affected members were found in the other families. These family pedigrees were consistent with autosomal dominant inheritance. Variable expression of the disease was noted in members within one family. Over pressure points punctate keratoses coalesced into hyperkeratotic plaques. There was palmoplantar hyperhidrosis in 3 families associated with keratosis. Continuous systemic retinoid treatment can clear symptoms. Future genetic classification on a molecular basis may reveal the existance of more than one entity of this clinically heterogeneous genodermatosis.

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Effect of Indigenous Herbal Drug Ficus Carica In-Patients with Vitiligo

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The aim of the study was to evaluate efficacy and tolerability of powdered extract of Ficus carica 250 mg (FC250) in the treatment of vitiligo. The incidence of vitilago was investigated by studying all the patients who were called at Sohana Eye Hospital near Chandigarh during a specific day. The patients were examined thoroughly and it took 3 doctors and 5 h to complete the program. Vitiligo was present in 28 patients and majority was from rural background. Females in age group of 25 years were predominating. VDRL tests were performed to exclude syphilis. They were directed to look for any local reaction, itching or blister formation and consult the same doctors after 10 days for routine checkup. Patients were allocated to receive FC250 for a period of 8 weeks. Paste of the drug for local application was also recommended. The patients were directed to expose the hypopigmented patches to sunlight for 2 min, after internal administration of FC250 and local application of the drug. The response to therapy was evaluated at time interval of 8 weeks by calculating leucoderma score [L.S] and pigmentation index [P.I]. 23 out of 28 patients attended the 2nd phase of the clinical trial after 8 weeks. 8 patients showed 15–33% improvement, 5 patients showed 35–60% improvement and 10 patients showed less than 25% improvement. Thus it was concluded that 30% of the patients showed significant improvement in development of pigmentation. Slight itching and blister formation was reported in couple of cases but reduced after discontinuing the treatment. Gastritis, dry mouth and headache were common complaints. Liver function tests were performed on regular basis and it was concluded that the drug was free from hepatotoxicity. In case of efficacy it was concluded that results shown by the herbal drug were in comparison to Psoralia corylifolia and Ammi majus (sources of Psoralen and Xanthotoxin), but it often failed to bring back complete normal color of the skin in depigmented area. Ficus carica is important medicinal plant of Ayurveda and finds application in formulations fro the treatment of leucoderma. Because of this further investigations into the active constituent of Ficus carica and role in the treatment of vitiligo must be undertaken.

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Uveal Melanoma – Adjuvant Therapy with Interferon Alpha 2 b

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Uveal melanoma is a rare entity of malignant melanoma compared to cutaneous malignant melanoma. It carries a significant risk of recurrence and metastatic spread. We examined the potential effects of adjuvant interferon α 2 b. 39 patients (23 male and 16 female patients, mean age 56.5 years, range 35–78 years) with uveal melanoma encountered between June 1996 and December 2001 received adjuvant treatment with interferon α 2 b 3 million units 3 times a week over a period of one year. All patients had therapy of the primary tumor before interferon treatment: Twelve patients had enucleation, 13 patients had Leksell Gamma Knife radiosurgery, nine patients had a Ruthenium 106 applicator, four patients had transpupillary thermotherapy (TTT), and one patient had ruthenium applicator combined with transpupillary thermotherapy. History, potential risk factors, clinical presentation, therapy side-effects and clinical course were documented. Outcome was compared with that obtained in 72 patients with uveal melanoma (33 male and 39 female, mean age 64.7 years, range 40–92 years) encountered during the same period, who did not receive adjuvant treatment. Among all patients, local recurrence occurred in 27 patients (24.3%), distant metastases in 20 patients (18.0%) and 17 patients (15.3%) died due to disseminated melanoma during the study period. 5 years survival rate was 49% for the interferon-treated group and 80% for the noninterferon-treated group (log rank test: not significant). In a multivariate approach, horizontal tumor diameter was the most significant prognostic parameter. Again, interferon therapy showed no significant effect in multivariate analysis. There was, however, a trend towards unfavourable outcome in the interferon-treated group. Our data show no evidence for a protective effect of adjuvant interferon treatment in ocular melanoma. Though the study was not randomised, the negative trend in the interferon-treated group in a multivariate approach suggests that at the moment interferon treatment cannot be recommended for uveal melanoma unless other clinical trials will reveal more favourable results.

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Treosulfan and Gemcitabine in Metastatic Uveal Melanoma: First Multicenter Experience

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Background: No effective treatment currently exists for metastatic uveal melanoma. However, recent results obtained by an ATP-based tumor chemosensitivity assay (ATP-TCA) have shown consistent activity of treosulfan + gemcitabine in 80% of tumor specimens tested. In this study we compare first clinical results observed with this drug combination at different European centers in patients with metastatic uveal melanoma. Methods: The study was a clinical case series of patients with metastatic uveal melanoma treated with treosulfan + gemcitabine at seven separate centers. Fourteen patients, 13 previously untreated and 1 pretreated with chemoimmunotherapy, were included in the study. Patients received treosulfan + gemcitabine in four different dose regimens. The response rates, progression-free and overall survival, and toxicity were evaluated. Results: The analysis of 14 patients revealed 1 complete response, 3 partial responses, and a stable disease in 8 cases. The objective response rate was 28.6 %, the median overall survival was 61 weeks (95 % confidence interval, 54 – 133 weeks), the progression-free survival was 28.5 weeks (95% confidence interval, 13- 62 weeks), and the one-year survival rate was 80%. The drugs were well tolerated. The most common side effect was leuko- and thrombocytopenia. Conclusions: These preliminary results suggest the potential therapeutic benefit of treosulfan + gemcitabine treatment in metastatic uveal melanoma and warrant further controlled studies.

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Influence of Cladribine on Normal and Diseased Human Skin Fibroblasts and Human Leukaemic Mast Cell Line

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Single clinical observations both in literature data and our own seem to be in favour of 2-chlorodeoxyadenosine (2-CdA, cladribine) use in systemic mastocytosis patients. So, the aim of our study was to examine susceptibility of human leukaemic mast cell line (HMC-1), skin fibroblasts from the lesions of urticaria pigmentosa (UP), psoriasis vulgaris (PsV), scleroderma (Scl) and normal skin towards 2-CdA. Cells, either HMC-1 line or skin fibroblasts from the third passage were stimulated with different concentrations of 2-CdA (0.1; 1.0; 2.5; 5.0; 10.0 and 20.0 nM/ml) for 24 or 72 h. Proliferation assays were performed in quadruplicate. After 24- h-incubation, we observed proliferation inhibition of all the studied cells in a dose dependent manner. HMC-1 cells were slightly less susceptible than normal skin fibroblasts to 2-CdA inhibition (at 20.0 nM per ml – proliferation decrease by mean 52.0 vs. 60.2%, respectively). However, surprisingly in all studied cultures, after a single dose of 2-CdA, followed by 72- h-incubation-period, considerable proliferation increase, independent of 2-CdA dose, ranging from 49.1 to 136.8% was observed. This effect was abolished when 2-CdA was added repeatedly to the cultures every 24 h. We noticed that cladribine seems to be more cytotoxic towards normal skin fibroblasts than UP ones – at 20.0 nM per ml of 2-CdA – proliferation decrease by mean 60.2 vs. 26.8%, respectively. Scl fibroblasts were much less sensitive to 2-CdA than normal, PsV and even UP ones – at 20.0 nM per ml of 2-CdA – proliferation decrease by mean 20.5, 38.9, 26.8%, respectively. We conclude that in order to sufficiently suppress cell proliferation, repeated doses of 2-CdA, added to cell cultures every 24 h are required. As for clinical application in systemic mastocytosis, this new purine nucleoside analog should be carefully considered as regards risk-benefit ratio.

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Skin Problems in Paediatric Transplant Recipients

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Skin diseases are frequent in organ transplant recipients but studies about children are sparse. Pediatric organ transplantation accounts for 5–10% of all transplantations. The aim of this work is to present skin diseases in children after solid organ transplantation and to compare them with lesions occurring in adult transplant patients. Our experience concerns 170 children out of a registry of more than 2000 transplant patients referred to our department during the past 10 years either for a systematic examination (all kidney graft recipients) or for a specific disorder (all the nonkidney ones). They included 133 kidney, 17 liver and 20 heart transplant recipients. The mean age at transplantation was 9 years and the majority of patients were under triple immunosuppressive therapy comprising corticosteroids, azathioprine and cyclosporine. More then 40 children had also one of the new immunosuppressive drugs (tacrolimus and/or mycophenolate mofetil) that were introduced either initially or after switching. Transplanted children frequently present side-effects of immunosuppressive drugs and infectious diseases. Steroid-induced striae distensae and acne were seen only in adolescents. Severe cyclosporine-related side-effects were more frequent in younger children. No specific side-effects were seen in patients taking tacrolimus and mycophenolate mofetil. The commonest findings were warts (55%), tinea versicolor (15%), herpes simplex/zoster (10%), molluscum contagiosum (7%), impetigo contagiosum and folliculitis (6%). Other notable disorders included a diffuse hyperpigmentation with a ''dirty'' appearance of the skin, pyogenic granulomas, proliferation of melanocytic nevi and skin tags. Skin cancers were not observed in children but developed in early adulthood. Three of 28 further adult patients who were transplanted during childhood had squamous cell carcinomas. Most disorders are related to the age of the patients rather than to the length of immunosuppression whereas others are due to the reinforcement of immunosuppression.

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Basophil CD63 Surface Expression Assay – A Useful Functional Method in Chronic Urticaria

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The aetiology of chronic urticaria is still unknown. Currently the concept has evolved that the chronic idiopathic urticaria (CIU) group might be autoimmune in origin, since it has become clear that 27–50% of these patients have functional autoantibodies directed against the α-chain of the high affinity IgE receptor (FcεRIα). These antibodies are identified by autologous serum skin testing and confirmed by histamine release studies, immunoblot or ELISA methods. Our purpose was first to measure the percentage of patients with autoimmune chronic urticaria with two methods. We applied the autologous serum skin test and a new assay for the detection of the surface expression of the activation marker CD63 on basophils. Finally we compared the results of the 2 methods and were looking for correlation. Autologous serum skin testing were carried out according to the literature. Surface expression of the basophil activation marker CD63 was measured on buffy coat without any previous activation. Phycoerythrin-conjugated anti-CD63 and FITC-conjugated anti-IgE monoclonal antibodies and flow cytometry was used. We measured the percentage of CD63 positive cells within the IgE positive cell population. The buffy coat cells were obtained from atopic donors with extrinsic atopic dermatitis and allergic rhinitis. 60.3% of the sera were positive in the autologous serum skin test and 56.8% in the CD63 expression assay. We obtained some correlation between the serum skin test results and the CD63 expression assay.

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Imiquimod for the Treatment of Cutaneous T Cell Lymphoma

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Cutaneous T cell lymphomas (CTCL) are defined as malignant clonal proliferation of skin-infiltrating T cells. In most CTCL, neoplastic cells express type 2 cytokines and are attacked by type 1 cytokine expressing reactive lymphocytes. Immunmodulators which induce type 1 cytokines (i.e. IFN-α) have been shown to be effective in CTCL treatment. At this background, imiquimod (1-(2-methylpropyl-)-1H-imidazol[4,5-c]-quinolin-4-amine), an immunmodulator inducing IFN-α, IFN-γ and IL-12 but down-regulating IL-4 and IL-5, has been applied as 5% ointment for the treatment of CTCL and CTCL precursors in two phase 1/2 studies. Firstly, 7 patients suffering from mycosis fungoides (MF, n = 5) and parapsoriasis en plaques (PEP, n = 2) were treated with alternating administration of imiquimod at 5 lesions located at different skin regions. Application was carried out 5 times per week for at most 6 months with reduction to 3 times per week in case of local erosions. Outcome of the treatment was assessed by the overall response. Complete remission (CR) was observed in 3 cases (PEP, n = 2; MF, n = 1), partial remission (PR) was found in 3 MF patients, whereas 1 patient with tumour stage MF showed progressive disease. In the second study, 10 patients suffering from MF stage IA-IIA were treated by application of imiquimod at 1 lesion of 20 cm^2. Administration was carried out 5 times per week for 12 weeks with reduction to 3 times per week in case of local erosions. Outcome of the treatment was assessed by the overall response and by evaluation of index lesions. Response was observed in 2 cases (1 CR, 1 PR), whereas 5 patients showed stable or progressive disease. Therapy was discontinued in the remaining 3 patients (withdrawal of consent in 2 cases, arrhythmia in 1 case). In contrast to the overall response, treated lesions disappeared completely in 4/7 patients. Adverse effects included local irritation (n = 5) or erosion (n = 3), fever (n = 2), nausea (n = 2), and arrhythmia (n01, study discontinuation). In summary, topical imiquimod can be successfully applied in MF and PEP. This treatment is well tolerated and applicable in the outpatient care. Due to an insignificant systemic effect, at least one lesion per skin region should be treated to attain a sufficient response in CTCL.

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Further Study of HHV6 Reactivation in Hypersensitivity Syndrome

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Hypersensitivity syndrome is a severe adverse drug reaction caused by the specific drugs including anticonvulsants, salazosulfapyridine and allopurinol. The clinical symptoms are high fever, multiple lymphadenopathy, severe skin rash, mononucleosis, and multivisceral involvement, which appear two to six weeks after the beginning of drug administration. Moreover, it is well known that relapse of symptoms, such as fever, eruption, and hepatitis, is observed several weeks after discontinuation of causative drug treatment in this syndrome. We presented that reactivation of HHV-6 was significantly associated with hypersensitivity syndrome at the 4th International Congress on Cutaneous ADRs. We examined additional patients to further characterize the hypersensitivity syndrome–HHV–6 interaction. To clarify the relationship between clinical manifestations and HHV-6 reactivation, we performed quantitative real-time PCR assays of serial serum and blood samples, as well as measurements of the anti-HHV-6 IgG titers. As a result, we clearly demonstrated the recurrence or worsening of symptoms such as hepatitis and fever was observed concurrently with HHV-6 reactivation. Our findings suggest that severe hypersensitivity syndrome is a complex pathological condition that involves both drug allergy and HHV-6 reactivation.

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Melanocortin 1 Receptor Accounts for 50% of Variation in Hair Melanin Eumelanin and Phaemelanin in a Northern European Dataset

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The melanocortin 1 receptor (MC1R) is a major determinant of physiological variation in pigmentation in many world populations. Previous studies have used a simple Mendelian model to relate genotype to phenotype. In the present study we have attempted to move beyond this simple framework to approach the subject as a quantitative issue: what proportion of variation can be explained by allelic changes at MC1R? What proportion of variation is unexplained and must be determined by othe loci? We studied 79 individuals comprising 38 volunteers with red hair and 41 nonred headed subjects. MC1R status was determined using automated sequencing. Known functionally significant changes at codons 142, 151, 160, 294 and frameshifts were counted as mutant: other changes were treated as wild-type/pseudo-wildtype. Hair melanins were assayed for eumelanin (PTCA) and phaeomelanin (AHP) using HPLC methods, including a recent modification enabling more direct estimation of phaeomelanin (AHP)levels (in press). Hair colour was classified according to the L'Oreal hair colour standards chart, and by using trispace L*a*b* scores, using a chromometer, were obtained. As expected, there was a decrease in red hair colour and phaeomelanin products with age. Using ANOVA models we showed clear differences between the groups with strong evidence for a MC1R heterozygote effect ($p < 0.01$) in determining eumelanin and phaeomelanin amounts and the ratio of phaeoelanin to eumelanin. MC1R gene status predicts hair colour and accounts for about 50% of the variation in this particular data set. Our results, show for the first time, that objective measures using Laboratory colour, particularly the a* and b* index ($p < 0.01$) may well be preferable to classifications based on subjective hair colour charts.

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A New Objective and Validated Method to Measure Itch in Children with Atopic Dermatitis

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Itch is a major symptom of skin disease. We wished to develop an objective method for measuring itch (as scratch) that could be useful in clinical trials and to monitor disease activity. Our strategy has been to use wrist-worn accelerometers, and to validate the use of such accelerometers in patients in their own homes against infrared videoing of people. We have studied 21 subjects (14 with atopic dermatitis and 7 controls, age range 2–9). We used accelerometers purchased from Cambridge Neurotechnology, worn on each limb, and subsequently interfaced via a proprietary reader to a PC for analysis. Ethograms of child behaviour were made from direct observation of infrared videoing carried out in the patient's own house. Child scratching behaviours were highly heterogeneous and comprise up to 5% of the time in bed (compared with 0.2% in controls, $p < 0.01$). Scratching and restlessness, as operationally defined, show a high degree of covariance ($p < 0.01$ $r^2 > 0.9$). There were clear differences for most of the variables measured between atopic children and controls. We developed a number of algorithms to analyse the digital output from the accelerometers including counting epochs with no movement, or simple double integration of scores. Whichever analysis was used, clear differences were evident between atopics and the controls ($p < 0.01$). There were no systematic differences between left and right, and whereas there is an interesting interaction between leg and arm scores, it is likely that only one limb accelerometer may need to be used in future studies. Finally, we note that the objective scores did not correlate with subjective parental VAS scores. In conclusion we have developed a new objective measure of disease activity useful for studying children with atopic dermatitis that has a clear role both in studying itch physiology and as an endpoint in clinical trials.

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Epidemiological and Clinicopathological Features of Basal Cell Carcinomas (BCC) Developing in Organ-Graft Recipients (OGR)

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OGR are at increased risk for developing various cancers, namely cutaneous squamous cell carcinomas (SCC). Although the incidence of BCC is also increased in comparison with the general population, these tumors have not yet been extensively studied in OGR. We studied the epidemiological and clinicopathological features of BCC developing in OGR (OGR-BCC) in comparison with an equivalent number of BCC retrieved randomly from the files of our Dermatopathology laboratory, obtained from a control group (C) of nonimmunosuppressed patients (C-BCC). 145/2028 OGR seen in our clinic developed 170 BCC. 40/177 OGR who developed over 600 SCC had also at least one BCC (SCC:BCC ratio 4.3:1). OGR-BCC appeared on average 6.94 (± 4.7) year after transplantation. The men:women ratio was higher in OGR-BCC than in C-BCC (4:1 vs 1.3:1, p<.001), and this predominance remained significant (p<.001) after adjustment for the overrepresentation of men (68%) in the group of OGR. The mean age at diagnosis of BCC was significantly lower in OGR as compared with the C (55±1 vs 70±11,5 yrs, p<.01). BCC were predominantly located over the head & neck, more frequently on the ear in OGR as compared with C (p<.05); moreover some unusual localisations (genitalia, wrist, hand) were found in OGR. Histologically, the mean thickness (Breslow) of OGR-BCC (1.1 ± .8 mm) did not differ significantly from that of C-BCC (1.35 ± .9 mm). Superficial type BCC were more frequent in OGR (34%) than in the C (14%, p<.001). Epidermal ulceration was found in 51% of OGR-BCC and 44% of C-BCC (NS). The density of the peritumoral cell infiltrate (scored semi-quantitatively) was significantly lower in BCC-OGR as compared with C-BCC (p<.001). Our data confirm that in OGR, BCC appear at a younger age, affect predominantly men, and can be observed in unusual localisations; histologically, they tend to be superficial, probably because they are diagnosed at an earlier stage. The reduced inflammatory peritumoral reaction in OGR-BCC is probably due to the chronic immunosuppression.

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Is Lichen Myxoedematosus an IgG-Mediated Disease?

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Lichen myxoedematosus is a rare, slowly progressive disease characterised by dermal acid mucopolysaccharide, predominantly hyaluronic acid deposits. Paraproteinemia is often observed. Its pathogenetic significance is however, not clear. We treated a patient with selective IgG ablation by immunoapheresis to study the influence of IgG in disease activity. We describe a 44-years-old lady with a nine year-history of increasing papular lichenoid lesions on forearms, knees and face. Histology revealed Lichen myxoedematosus. A monoclonal IgG (κ) could be demonstrated on serumelectrophoresis. A plasmocytoma could be excluded. As the patient did not agree to melphalan therapy, we instituted plasmapheresis for eight cycles. This therapy induced a slight improvement of the skin lesions. Immunoapheresis employing a Ig-specific-column (Ig-Therasorb™, Plasmaselect) was performed for 30 cycles at appr. one week intervals. Removal of the IgG paraprotein was efficient. Before treatment the paraprotein formed a 17.9% fraction at a total protein of 78 g per l, i.e. 13.96 g per L. Before the last immunoapheresis the paraprotein was 10.3% of 60.1 g per l, i.e. 6.19 g per L. We measured the skin relief at 10 standardised sites by visiometer. The wrinkle depth increased from 0.1 mm to 0.3 mm 20 MHz-ultrasound documentation of the skin structure demonstrated a decrease in the amorphous dermal infiltrate. While plasmapheresis removes a plethora of serum proteins, immunoapheresis with the applied column removes IgG, as well as some IgM and IgA, but no other serum proteins. Thus the treatment success in our patient supports the notion that the paraprotein commonly observed in Lichen myxoedematosus has a pathogenetic role in this disease.

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Increased Sp1 phosphorylation as a mechanism of hepatocyte growth factor (HGF/SF)-induced transcription of vascular endothelial growth factor (VEGF/VPF): contribution of the PI-3 kinase/Akt and MEK1/2 signaling pathway

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Hepatocyte growth factor (HGF/SF)-induced expression of vascular endothelial growth factor (VEGF/VPF) has been implicated in paracrine amplification of angiogenesis, contributing to angiogenic responses during inflammation, wound healing, collateral formation and tumor growth. We have shown previously that HGF/SF-mediated VEGF/VPF expression by keratinocytes is primarily dependent on transcriptional activation, mapping the HGF/SF-responsive element to a GC-rich region between bp –88 and –65. Sp1-like factors bind to this element constitutively, however the VEGF/VPF promoter is transactivated by HGF/SF in the absence of induced binding activity. In experimental approaches to clarify molecular mechanisms of Sp1-dependent VEGF/VPF gene transcription, neither HGF/SF-dependent changes in nuclear expression nor in relative DNA binding activity of Sp family members to the indicated element were observed. Thus, HGF/SF was hypothesized to induce VEGF/VPF gene transcription via increased transactivation activity of Sp1 due to biochemical modification. In immunoprecipitation studies, HGF/SF was found to increase the amount of serine-phosphorylated Sp1, revealing a likely mechanism of HGF/SF-induced VEGF/VPF expression, as phosphorylation may enhance of Sp1 transcriptional activity. By the use of chemical inhibition, overexpression of kinase-deficient signaling proteins, and transfection of antisense oligonucleotides, we herein provide evidence that PI-3 kinase/Akt, PKC-zeta, and MEK1/2 are essential for HGF/SF-induced VEGF/VPF promoter activation. Together, our results elucidate a critical pathway of paracrine amplification of angiogenesis, suggesting that HGF/SF-induced Sp1 phosphorylation may activate VEGF/VPF promoter activity, requiring the contribution of distinct pathways.

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Impaired Dihydropterin Reductase Activities (DHPR) in Patients with Vitiligo

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The essential cofactor for the hydroxylation of the amino acids L-phenylalanine, L-tyrosine and L-tryptophan is (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$). $6BH_4$ is synthesised *de novo* by the conversion of GTP via GTP-cyclohydrolase I or by regeneration of the reduced $6BH_4$ via pterin carbinolamine dehydratase (PCD) and DHPR. DHPR is an NADH dependent reductase that converts quinonoid dihydropterin into $6BH_4$. The method for the determination of DHPR activity in whole blood samples uses oxidised cytochrome C to produce reduced cytochrome C that is measured at 550 nm. Our investigation compared DHPR activity in blood samples from healthy controls and patients with the depigmentation disorder vitiligo. The results yielded significantly increased activities of DHPR in patients with vitiligo compared to controls. Since there are two pathways for the supply of the essential cofactor $6BH_4$ it could be possible that the recycling process contributes to the pool of reduced $6BH_4$. However, it has been shown that patients with vitiligo have a pathological increased shortcut to quinonoid dihydropterin due to the deactivation of PCD by hydrogen peroxide. Hence increased production of quinonoid dihydropterin may induce DHPR explaining the elevated activity measured in this patient group.

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Treatment of Primary Cutaneous B-Cell Lymphoma with Intralesional Rituximab®

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Rituximab® is a genetically engineered antibody directed against the CD20 antigen. Intravenous administration of rituximab® has been used for the treatment of patients with low-, intermediate-, and high-grade B-cell non-Hodgkin lymphomas, and is a registered treatment modality for this indication. Treatment of cutaneous B-cell lymphoma (CBCL) with intralesionally applied rituximab® has been described in a few cases. We report on 6 patients with CBCL (M:F = 3 : 3; mean age: 64,7; range: 48–80; follicle center cell lymphoma-FCCL: 3, marginal zone lymphoma-MZL: 3) that have been treated with intralesional administration of rituximab®. Rituximab® was applied intralesionally according to the following scheme: each patient received 20–30 mg of rituximab®/application, fractioned in one or more doses depending on the number of lesions treated (minimal single dose: 10 mg). The applications were repeated 3 times over a period of one week. A new cycle of treatment was repeated after 4 weeks if lesions failed to show complete clinical response. Complete remission could be observed in 4 patients (FCCL: 2; MZL: 2) after 1, 2, 6, and 7 cycles of treatment, respectively. Clinical remission was verified by histologic examination. Two patients (FCCL: 1; MZL: 1) achieved only a partial remission after 4 cycles of treatment, and are currently being treated. No severe side-effect occurred except for slight pain during injection. FACS analysis of peripheral blood revealed complete disappearance of B-lymphocytes for a period of few weeks after application, without clinical relevance. In conclusion, intralesional rituximab® therapy is a well tolerated and effective treatment for CBCL. Response to therapy, however, is different for different patients, and several cycles of treatment may be necessary to achieve complete response.

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Comparative Time Courses of Blood Flow and Itch in Wheal-and-Flare Reactions Induced by Histamine and Type I Allergens

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As part of ongoing investigations into the perception of itch, we have conducted a clinical study to more fully characterize the physiological response to skin prick testing. Cohorts of atopic and nonatopic individuals (n = 14) underwent a standardized prick test procedure using histamine, saline control and the relevant type I allergen (grass pollen/house dust mite; atopics only) during the winter months. Skin blood flow was measured continuously by laser Doppler flowmetry over a 20-min period, with the volunteers' concurrent perception of itch intensity being recorded using an electronic visual analogue scale. Analysis of the data revealed significant differences between the responses to histamine and allergen in atopic subjects. Histamine induced a more rapid increase in blood flow, which occurred in parallel with the onset of itch. However, the magnitudes of both blood flow increase and itch intensity were significantly greater following allergen administration and, unlike the histamine-induced changes, were sustained at their maximum levels throughout the 20 min assessment period. Atopics and nonatopics exhibited similar responses to histamine. The study lends support to the view that histamine is only one of a number of mediators responsible for the allergen-induced wheal-and-flare response, and also indicates that prick testing of sensitized individuals with the appropriate allergen provides a better paradigm for the sensation of itch than histamine alone.

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Alefacept Selectively Reduces Subpopulations of Memory $CD4^+$ and $CD8^+$ T Cells

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Activated memory ($CD45RO^+$) T cells are believed to mediate the inflammatory process in psoriasis. Alefacept (human LFA-3/IgG_1 fusion protein) has been shown to reduce circulating levels of $CD4^+$ and $CD8^+$ memory T cells. An open-label study of patients with chronic plaque psoriasis was designed to assess the mechanism of action of alefacept and its effects on subpopulations of memory T cells. Alefacept 7.5 mg was administered once weekly by IV bolus for 12 weeks. Immunohistology and flow cytometry were used to study T-cell populations in skin lesion biopsies and peripheral blood. Data are available for 13 patients at this time. At week 13, 8 patients (62%) achieved histologic remission and were judged complete responders, with reversal of K16 expression in the epidermis and reduction of epidermal hyperplasia; 5 patients were either partial responders or nonresponders. Mean PASI in all patients was reduced from 21.2 at baseline to 8.45 at Week 13, representing a mean decrease of 61%. From baseline to Week 13, 5 of 13 patients achieved $\geqslant 75\%$ PASI reduction; maximal PASI improvement occurred between Weeks 13 and 19, with 9/13 patients achieving this level of response. Mean epidermal thickness was significantly reduced in responders at week 13. In complete responders, there were mean reductions of 81% for epidermal T cells, 70% for epidermal $CD8^+$ cells, and 56% for epidermal $CD103^+$ (epithelial homing) cells. Thus, circulating $CD8^+$ memory and $CD8^+$ epithelial homing T cells are affected to the largest extent by alefacept. These populations are major T-cell subsets found in the epidermis of lesional psoriatic skin. At week 13, epidermal thickness was highly correlated ($r = 0.87$) with the residual number of T cells in the epidermis of lesions. In responders, the reduction in epidermal T cells (averaging 27×10^9 cells) far exceeded the reduction of circulating memory T cells (averaging 3.4×10^9). The reduction from baseline in IFNγ-producing T cells in the circulation (57%) exceeded that of the overall memory T-cell population (30%), supporting the selectivity of alefacept for Type 1 T cells. These results establish selective effects of alefacept on disease-related T cells with at least 8:1 therapeutic selectivity. These data are most consistent with selective depletion as the relevant therapeutic mechanism of alefacept, though relative immune deviation may also contribute to efficacy.

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Retreatment with Alefacept Improves Clinical Response: Results from Two Phase III Trials

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The efficacy and safety of alefacept in patients with chronic plaque psoriasis has been evaluated in two randomized, placebo-controlled phase III trials. Alefacept 7.5 mg was administered by 30-second IV bolus in 1 trial and as a 15-mg intramuscular (IM) injection in the other. A course of treatment was defined as once-weekly injections of alefacept for 12 weeks followed by 12 weeks of observation. A single course of IV or IM alefacept provided statistically and clinically significant reductions in the symptoms and severity of psoriasis compared with placebo. Two courses of alefacept provided greater benefit in both studies. Patients who received two courses of alefacept (7.5 mg IV or 15 mg IM) had a maximum reduction from first-course baseline Psoriasis Area Severity Index (PASI) of 54% at 6 weeks after the last IV dose and 45% at 12 weeks after the last IM dose of the second course. The percentages of patients who achieved ≥75% reduction in PASI during the entire study period after a single course and after a second course were 28% and 40%, respectively, for alefacept IV, and 33% and 43%, respectively, for alefacept IM. Corresponding response rates for ≥50% reduction in PASI were 56% and 71% for alefacept IV and 57% and 69% for alefacept IM. The percentages of patients who achieved ''clear'' or ''almost clear'' by Physician Global Assessment after two courses of therapy were 32% and 31% for IV and IM alefacept, respectively. No rebound of disease or flares were observed following treatment cessation in either study. Notably, repeated courses of IV and IM alefacept were well tolerated, with similar adverse event reports during Course 1 and Course 2. There was no evidence of a cumulative effect on lymphocytes. In conclusion, two courses of IV or IM alefacept produce significant clinical improvements in psoriasis. The incremental effectiveness of a second course of alefacept provides strong support for its use as an intermittent therapy.

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Skin Problems in Adult Organ Transplant Recipients

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The number of organ allograft recipients worldwide is steadily increasing and the chronic immunosuppressive therapy necessary for graft function frequently results in cutaneous side-effects. In addition to the direct side-effects of drugs (e.g. acne vulgaris, hirsutism, skin atrophy, gum hypertrophy), infections and malignancies are the most commonly encountered problems in adults. Fungal and viral infections, particularly by the human papillomaviruses (HPV), may be extensive and refractory to treatment. Non-melanoma skin cancers are the commonest post-transplant malignancy, with the risk for SCC increased 50–100 fold and BCC up to 10-fold. These tumours are often multiple, aggressive and have increased metastatic potential. Ultraviolet radiation and reduced immunesurveillance clearly play a role in their pathogenesis, but there is mounting evidence implicating an important cofactor role for HPV, particularly by viruses of the epidermodysplasia verruciformis subgroup. Predisposing genetic factors such specific polymorphisms in p53 and GST may also be relevant. Malignant melanoma, cutaneous lymphoma and Kaposi's sarcoma are over-represented and evidence is emerging that rarer appendageal tumours, especially those of sebaceous gland origin, are also more common. The diverse spectrum and high frequency of cutaneous malignancies observed in organ transplant recipients highlights the importance of regular surveillance of this high-risk population and the need for well-designed preventative and therapeutic management strategies.

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Eczematous Dermatitis During Treatment of Hepatitis C with Interferon α and Ribavirinin

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Introduction: Interferon α (IFNα) and ribavirin combination therapy is currently considered the most effective treatment for chronic hepatitis C. Both monotherapy with IFNα, and combination treatment have been associated with an increased prevalence of inflammatory skin diseases. Background: Cutaneous adverse effects may be an obstacle to adherence with long-term combination therapy of chronic hepatitis C. A better characterization of cases is needed in order to define the role of a dermatologic approach in concerned patients. Observations: Between january 1999 and april 2002, 53 patients receiving combination treatment with ribavirin and interferon alfa were followed prospectively. Eight patients developed *de novo* generalized eczematous dermatitis. One of them had a personal history suggestive of atopic diathesis. A search for elevated specific IgE (phadiatop°) was performed in 5 patients, and was positive in 3 patients. One patient noted an aggravation of a preexistent eczematous dermatitis. One patient developed a localized nummular eczematous plaque distant from IFNα injection sites. First symptoms appeared between a few days, and 9 months after onset of treatment. Treatment with mid-potent topical corticosteroids and emmolients was effective in most patients; in no case had the combination therapy to be interrupted. Discussion: The observations suggest that eczematous dermatitis is a frequent side effect of combination therapy for chronic hepatitis C. At least a portion of cases is associated with elevated specific IgE, but further studies are needed to determine the role of atopy as a predisposing factor vs. a consequence of therapy with IFNα and ribavirin.

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Herpes Simplex History, Serology, and Symptomatic Infections in Patients Treated with Topical Tacrolimus for Atopic Dermatitis

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Introduction: Tacrolimus ointment is a new and effective treatment for atopic dermatitis. As a main advantage over topical corticosteroids, this macrolide immunosuppressor does not induce cutaneous atrophy. Topical tacrolimus ointment will therefore become a treatment of choice for sensitive areas such as the head and neck, which is also a site of predilection for herpes simplex infection. Background: Eczema herpeticatum is a potentially serious complication of herpes simplex infection, and has been observed during treatment of atopic dermatitis with tacrolimus ointment. Objective: To study symptomatic herpes simplex infections in a cohort of 52 patients with atopic dermatitis that were treated with tacrolimus ointment. Results: Overall, 19% of patients presented with symptomatic herpes simplex. None of the patients with a positive history and/or recurrent herpes simplex suffered a complication, while all three patients with eczema herpeticatum had a negative herpes simplex history. Discussion: Our observations suggest that patients with a negative herpes simplex history may be more at risk for eczema herpeticatum than patients with symptomatic recurrent herpes simplex. A speculative explanation for this apparent paradoxon would be that patients with a negative herpes history have unrecognized symptomatic herpes simplex infection, and thus might benefit from targeted information about herpes simplex in order to reduce the risk of treating herpetic lesions with topical tacrolimus.

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Helio (Photo) -thalassic Therapy and Bio-Stimulated Pigmentation in Psoriasis

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Psoriasis is chronic disease with unclear etiology, genetic predisposition and unpredictable evolution. Conventional therapy does not stop the relapses, has numerous side-effects, so it is serious psychological stress for patients, who affect their quality of life, and leads to hospitalizem. This is great challenge for developing new treatment strategy based on natural recourses. We tried to promote the method of photo (helio)-thalassic therapy with biostimulated pigmentation in ideal climate of Ohrid Lake and higher segment of near Galicica Mountain. This region with many sunny days, in period from April to October, is particularly good for such treatment. We treated group of 40 patients- volunteers (age from 5 to 39) where diagnosis psoriasis vulgaris was clinically established and by histology confirmed. Duration of the disease was between 3 months and 25 years. Ten percent (10%) of patients were psihologicaly traumatized by previous long-time treatment. Weekly dynamics of therapy was, in mountain segment once a week between 8 and 11 a.m., in lake segment every day between 8 and 11 a.m., and 3–6 p.m. For stimulation of pigmentation, 1 h before exposure to the sun locally was applied extracts from plants and bee products. State Health Institute previously approved their use. Medium duration of treatment was 3 weeks, and 32 (80%) of patients had complete remission (compared with 30% spontaneous remission of psoriasis) of the skin changes. These results are demonstrating and supporting bio-stimulated pigmentation in psoriasis vulgaris as additional therapy to conventional treatment.

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The Targeted Mechanism of Alefacept Selectively Reduces Memory-Effector ($CD45RO^+$) T Cells and is Related to Clinical Improvements in Psoriasis

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Alefacept is a fully human fusion protein consisting of the first extracellular domain of LFA-3 fused to the hinge, C_H2, and C_H3 sequences of IgG_1. The LFA-3 segment binds CD2 on the surface of T cells, while the Fc portion binds FcγRIII on accessory cells. These actions inhibit T-cell activation and proliferation and induce selective apoptosis of memory-effector ($CD4^+CD45RO^+$ and $CD8^+CD45RO^+$) T cells. These T cells are preferentially reduced because they express higher levels of CD2 than naïve ($CD45RA^+$) T cells. Memory T cells make up the majority of T cells found in psoriatic plaques and are thought to mediate disease activity in the skin. In randomized phase III clinical trials of alefacept in chronic plaque psoriasis, the relationship between memory T-cell reduction and clinical improvement was assessed. The magnitude of reduction in peripheral memory T cells was measured by the area under the effect curve (EAUC), and then divided into quartiles. Clinical response was measured by PASI and PGA. In a double-blind, international study (n = 507), patients were randomized to alefacept 10 or 15 mg or placebo weekly by IM injection for 12 weeks, followed by 12 weeks of observation. The percentages of alefacept-treated patients (10 or 15 mg) who had ≥75% PASI reduction from baseline at any time increased with increasing effect on peripheral memory $CD4^+$ T cells (21%, 28%, 33%, and 38% for EAUC quartiles 1–4, respectively). Corresponding percentages of patients who achieved PGA of ''clear'' or ''almost clear'' at any time were 13%, 16%, 29%, and 33%. Similar results were observed in a multicenter study (n = 553) that consisted of two double-blind, 12-week treatment courses, each with 12 weeks of treatment-free follow-up. Alefacept 7.5 mg and placebo were administered weekly by 30-second IV bolus injection. The percentages of alefacept-treated patients who had ≥75% PASI reduction from baseline at any time in Course 1 increased with greater reductions in $CD4^+$ memory T cells (16%, 20%, 32%, and 44% for EAUC quartiles 1–4, respectively). Of those receiving two courses of alefacept, corresponding response rates at any time in Course 2 were 19%, 9%, 30%, and 62%. The relationship between the extent of memory T-cell reduction and clinical efficacy supports a key role for this T-cell subset in disease pathology.

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The Role of Reactive Oxygen (ROS) and Nitrogen Species (RNS) in the Pathogenesis of Warts: Hypothesis and Reality of Clinical Trial

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The dozens of topical and systemic treatments of verruca vulgaris, hyperkeratosis caused by human papillomavirus, include chemical, cryo-, thermal, and photo-destruction, in combination with immunosensitization and topical application of pro-inflammatory agents. No treatment gives so far entirely satisfactory results, due to high incidence of relapses or nonresponders, morbidity, adverse effects, or low efficacy. In the last decade a ''double-edge sword'' role of ROS and RNS in the pathogenesis of viral infections has been hypothesized. The viral-induced low intracellular levels of ROS are triggers for nuclear transcription factor (NFκB), leading to increased proliferation of both the host keratinocytes and virus itself, while higher levels of ROS and RNS cause in a concentration-dependent manner either apoptotic or necrotic death of virus bearing cells, thus limiting the spread of infection to neighboring tissue. Although by different mechanisms, all the therapeutic agents used in the treatment of warts induce an increased production of ROS or/and RNS. After thorough laboratory investigation, a nutraceutical formulation (Immugen®, IDI Farmaceutici, Italy) containing inducers of intracellular ROS and RNS production (L-methionine and soy phospholipids) to eliminate virus-bearing cells, and strong antioxidants to protect the host cells against free radical destruction (α-tocopherol, ubiquinone, selenium aspartate) has been chosen. We performed a double blind placebo- and case-controlled randomized clinical trial (number of patients –59, selected for the presence of at least 1 relapse after cryotherapy; duration of treatment –180 days; number of warts per patient – > 10), comparing a combination of cryodestruction and oral administration of Immugen® and cryosurgery + placebo. The study was conducted with external monitoring, and in compliance to Good Clinical Practices. Results showed the statistically significant increase in total clearance of the virus (57% vs. 33% for placebo group), lower incidence of relapses (25% vs. 50%), good tolerability and compliance, and the absence of adverse effects. The interim analysis of results provides evidence that the administration of pro-/antioxidants in combination with cryosurgery can be recommended for safe and successful treatment of recurrent cutaneous warts.

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The Efficacy of 308 nm Excimer Laser in the Treatment of Psoriasis is Related to Individual Clinical Subtypes

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Objectives: The UV-light therapy of psoriasis using psoralen and UV-A (PUVA) or UV-B, especially 311 nm, is one of the most used treatments inducing clearance of psoriatic leasions. Recently, a successfully irradiation of psoriatic leasions with 308 nm Excimer Laser was described. This new type of treatment allows to aime at involved areas without hitting normal skin and uses more than 3-fold of the individual MED on the involed skin. Methods: To analize if all different types of psoriasis benefit to this new treatment and to standardize the therapy, a prospective study was performed: 60 patients (33 men and 27 women, mean age 49 years) where included in the study. The criterias of 1) persistance of psoriasis less or more one year, 2) the localization (e.g. scalp), 3) the involved body surface and 4) the clinical type (e.g. pustulosa) where related to number of Excimer laser treatments and the cumulative dosis of UV-B 308 nm until clearance was measured and compared to traditionally used 311 nm. Clinical improvement and relapse was documented by PASI over a time period of 4 months. Results: Patients with a short history of psoriasis < 1 year, especially with guttate-type and/or with several plaques profit extremly of Excimer laser treatment. In 36 patients who were treated 9,4 + 3.1 times with cumulative dosis of 9.8 J per cm^2 + 4,2 J per cm^2 , PASI was reduced from 9.3 + 4,2 to 2.1 + 1.8 . In contrast, the efficacy was less in patients with an involved body surface > 25% and with long history of thick chronic plaques and hereby high dosages of UV-B radiation (31.3 J per cm^2 + 6.7 J per cm^2) and many sessions 23.2 + 4.0 were necessary to reduce PASI from 15.3 + 3.1 to 8.3 + 2.7. The efficacy for treating scalp psoriasis (n = 5) was not convincing, but pustulosa psoriasis (n = 5) was cleared in 15.6 + 3.7 treatments using 16,9 J per cm^2 + 2.9 J per cm^2. Conclusion: Excimer laser generated 308-nm UV-B radiation is one of the most effective treatment modalities for moderate forms of psoriasis. Hereby cumulative UV-B dose is lower than seen in traditionally used 311 nm radiation. When applied as a monotherapy, UV-B 308 nm Excimer Laser therapy is less sufficient in the treatment of severe psoriasis with thick stationary plaques.

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Cutaneous Field Stimulation of Sensory Nerve Fibers Reduces Itch But Has No Effect on Experimental Delayed Reactions

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A new technique, cutaneous field stimulation (CFS), which electrically stimulates unmyelinated C-fibers, is used to treat itch in skin disease. The aim of this study was to investigate how experimental contact dermatitis responds to this treatment. Twelve patients with contact dermatitis to nickel used CFS for one hour during four days. A flexible plate with electrodes was applied on the test area on one upper arm and stimulated by a constant current (0.8 mA). On the fifth day they were provoked with nickel sulphate (allergic contact dermatitis) and benzalkonium chloride (irritant contact dermatitis) epicutaneously and with tuberculine (delayed immunologic reaction) intradermally. Twelve patients with type I allergy were treated with CFS on the lower arm for one hour and then pricked with histamine and the allergen extracts (the volume of the weals being measured) and tested with bensoic acid (nonimmunologic contact urticaria, closed test). Itch was recorded. In addition, 10 patients used CFS for four days and the experiments were performed on the fifth day. The allergic prick test reactions were increased after a single CFS treatment, the itch being significantly reduced after both single and repeated CFS treatments. All other test reactions remained unchanged. Repeated CFS seems to be a safe treatment of itch without adverse effects on contact dermatitis.

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Adverse Cutaneous Reactions to Imatinib (STI571) in Philadelphia Chromosome-Positive Leukemias: A Prospective Study of 54 Patients

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Background: Imatinib is a new major treatment in chronic myeloid leukemia. Objective: To study the cutaneous reactions induced by imatinib. Methods: All in- and out-patients with Philadelphia chromosome-positive leukemia, treated by imatinib were included in this prospective study. Clinical features, pathological findings, evolution of each case, and analysis of potential risk factors were recorded. Results: 54 patients were included, 48 of whom experienced at least one cutaneous reaction. These reactions consisted of 36 rashes, 35 edemas, and 22 pruritus. The rash was severe in 5 patients, resulting in temporary interruption of treatment in 3. Highly significant relationships were observed between the daily dose of imatinib and both rashes and edema. In a multivariate analysis, female gender and the daily dose of imatinib were independent risk factors for the development of rashes. Conclusion: Adverse cutaneous reactions induced by imatinib are frequent, generally moderate and dose dependent.

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Severity of Drug Reaction with Eosinophiilia and Systemic Symtoms (DRESS)

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The short and long-term prognosis of DRESS is poorly known. This was evalauted in a retrospective analysis of 55 patients hospitalized in a single center from 01 to 1995 to 06–2001. The severity of the disease was evaluated on 3 criteria: a severity score at diagnosis, duration of symptoms for more than 6 weeks, death. A diagnosis of DRESS was considered definite in 55 of 74 cases where this diagnosis had been proposed at discharge. The patients were 24 males and 31 females, the mean age was 45 years (range 15–84), 15/55 were of African ancestry (27%). The delay between onset of treatment and reaction was 31 days as a mean (1–79). Medications that were more often implicated were minocycline (11), carbamazepine (10), antibacterial sufonamides (8) and allopurinol (7). The severity score at diagnosis was above 6 in 17 patients, including 12 with black skin. (OR 28, 5.8–135) and 7 exposed to minocycline (0R 6, 1.4–24.5). The duration of the disease exceeded 6 weeks in 13 patients (24%) including 7 with black skin (OR 5, 1.3–19) and 7 exposed to minocycline (OR 11, 2.5–50). Five patients died (9%), including 2 with black skin. and 3 with a prolonged disease. These results confirm that DRESS is a severe disease with a high percentage of chronic forms. Black ethnicity and exposition to minocycline are factors predisposing to more severe and chronic disease.

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Thymic Index and Infantile Atopic Dermatitis: A Case-Control Study

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Atopic dermatitis has been hypothesized as the result of thymic dysmaturation. We studied thymic size in a cohort of children aged from one month to two years to check whether morphologic findings do support this hypothesis. Three groups of 20 children were enrolled: a group with atopic dermatitis, a group at high risk of atopy (infants with a first-degree parent suffering from atopic disease) and a control group. For each infant, a physical examination was performed and the size of the thymus was measured by sonography. In the group with atopic dermatitis, SCORAD index was calculated and IgE levels were obtained. No statistical difference in thymic index was found between the three groups. Within the atopic group, analysis of subgroups according to index of severity indicated the same trend, i.e. the higher the SCORAD index, the lower the thymic index. In this group, there was no relationship between IgE values and the thymic index. Confounders, such as systemic effect of topical steroids and possible biases in measurement are limiting factors in interpretation of these data. However, although these preliminary results need to be confirmed, the thymus may play a role in the pathophysiology of atopic dermatitis. The smaller size of the thymus in severe cases suggests that excessive apoptotic material from the thymocytes could be involved in the onset of atopic dermatitis.

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Validity of Self-Assessment of Melanoma Risk: Case-Control Study

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The aim of the study was to assess the validity of a risk stratification model for melanoma based on self assessment of risk factors. In a case-control study melanoma patients (n = 202) and controls (n = 202) matched by age and gender filled in a questionnaire including melanoma risk factors and were examined by specially trained interviewers. For analysing the data we used odds ratios of risk factors and compared a self-assessment based risk stratification model for melanoma with a model based on physician assessment of risk factors (ROC analysis). Self-assessment and physician assessment of risk factors identified the number of naevi, skin phototype, and UV-damage of the skin as independent risk factors for melanoma. ROC analysis showed no statistically significant difference between the accuracy of the self-assessment based risk model (area under the curve:0.73; 95% CI: 0.68–0.77) and the model based on physician assessment (area under the curve: 0.77, 95% CI: 0.73–0.83; p = 0.10). A group defined as being at high risk of melanoma derived from the self assessment based risk model and the physician based risk model included 39% (95% CI: 31%-48%) and 42% (95% CI: 33%-52%) of the melanoma patients while excluding 90% of the controls. A relatively simple risk stratification model based on self-assessment of risk factors is as accurate as a model based on physician assessment and could be useful for identification of a group of individuals at high-risk for developing melanoma.

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The EuroSCAR study

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A case control study of severe cutaneous reactions (SCAR) to drugs (Stevens–Johnson syndrome-SJS, toxic epidermal necrolysis-TEN and acute generalized exanthematous pustulosis-AGEP) was conducted in 7 european countries from September 1997 to December 2001. A total of 810 potential cases were collected prospectivelly. 443 cases of probable or definite SJS or TEN and 137 cases of probable or definite AGEP were enrolled together with 1589 controls. The results from preliminary case-control analyses confirmed that for SJS or TEN the drugs associated to the highest risks were anticonvulsants, oxicam NSAIDs, antibacterial sulfonamides and allopurinol. Compared with the results of a prior case-control study, the EuroSCAR study found a higher risk associated to the use of allopurinol and pointed to very high risks linked to two recently released drugs: lamotrigine and nevirapine. Lamotrigine, an anticonvulsant, was associated to a risk that was at least as high as those related to phenytoin or carbamazepine. Nevirapine, an antiretroviral agent took the place of sulfonamides as the main cause of SCARs in HIV infected persons. Concerning AGEP the EuroSCAR study confirmed the principal role of antibiotics as a cause of the reaction. Further analyses will precise the respective role of infections and antibacterial medications in inducing severe cutaneous reactions.

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Atopic Dermatitis and the Risk of Cancer

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Both blood and skin lymphocytes from adults with severe atopic dermatitis show telomere erosion and have increased telomerase activity similar to what is found in patients with lymphoma. Treatments with immunosuppressive agents such as tar, UV therapy and corticosteroids may also increase the risk of cancer. We therefore analyzed, if patients having severe atopic dermatitis have an increased risk of cancer. All individuals admitted with a primary diagnosis of atopic dermatitis during 1977–96 were identified in the Danish National Hospital Register and followed up in the Danish Cancer Register. A total of 6275 persons were admitted with atopic dermatitis during the 20-year period. Among 2030 adult patients an increased risk of cancer was observed; SMR = 1.5 (95%CI: 1.2–1.9). Half the excess cases of cancer was related to nonmelanoma skin cancer diagnosed within the first 10 years of discharge; SMR = 2.7 (95%CI = 1.6–4.3). Ten men developed nonmelanoma skin cancer vs. 3.6 expected; SMR = 2.8 (95%CI: 1.3–5.1). No increased risk of malignant melanoma nor lymphoma was observed and the cancer risk was not increased among women or children (No. 4245; age < 18 years). In conclusion, male adult atopic dermatitis patients had increased risk of nonmelanoma skin cancer but not other cancers. No increased cancer risk was observed among women and children.

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Skin Care in Organ Transplant Patients (SCOP) – Analysis of Risk factors, Prophylactical Strategies and Therapeutical Options Basing on the International Scop-Network

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Background: Transplantation of solid organs has been a well established mode of therapy for the treatment of various end-stage diseases for many years now. While in the first year following transplantation special emphasis is given to the prevention of viral, bacterial and fungal infections a significant increased incidence of non-melanoma skin cancer is paralleling the extended survival rates of grafted patients. Against the background of lifelong immunosuppression risk factors such as sun-exposure and infections with oncogene virus have to be discussed. Until now most of the published data contains only small groups of patients making comprehensive risk-factor analysis still difficult. Objective: The structure of an international, internet based, epidemiologic data-bank - the SCOP-Network - is presented. Results: First results highlight the significance of epidemiologic research in transplant medicine and emphasize the importance of standardized interdisciplinary and dermatological care in this field. Our data indicates that 40% of the organ grafted patients show premalignant or malignant lesions on their first visit. Further on 22% develop SCC, 17% BCC, 12% Bowen's disease and nearly 5% malignant melanoma. Actinic keratoses are found in 43% of these patients. 66% of the lesions are located on sun exposed areas of the skin. Various influencable riskfactors such as cumulative sun-exposure and the total immunosuppressive therapy burden are quantified and discussed against the innate individual susceptibility for skin cancer. Conclusion: The SCOP-Network represents a new type of multi national cooperation in the field of transplant-dermatology. Besides a epidemiologic databank, the integration of basic research and the exploration of new therapeutic options will provide effective strategies to ease the burden of skin infections and cutaneous malignancies in these group of patients. New data on the incidence of skin cancer related to the lifetime-sunexposure, the cumulative dose of immunosuppressive drugs or the load of oncogenous human papilloma viruses (HPV) might lead towards new preventive strategies.

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Accuracy of Drug Prescriptions and Risk of Toxic Epidermal Necrolysis

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One third of admissions for a drug adverse effect in an American hospital were secondary to an inappropriate prescription. Our aim was to check whether this was also true for toxic epidermal necrolysis (TEN) and Stevens Johnson Syndrome (SJS). In a retrospective series of 275 patients hospitalized between 1987 and 2000, we selected the 132 cases who had clear data on: (1) causative drug(s) belonging to the category of anticonvulsants, antibacterial sulfonamides, allopurinol, or oxicam NSAIDs, and (2) indication for prescription of this causative drug. The prescription was judged questionable when (1) indication was different from the designated use or (2) precautions or contraindications were not respected, both as labelled in the 2001 formulary. According to the 2001 recommendations 76 prescriptions (58%) were questionable: 24/32 (75%) for trimethoprim-sulfamethoxazole (mild urinary tract infection), 0/25 (0%) for sulfadiazine, 4/4 for sulfadoxine 9/20 (45%) for carbamazepine (acute pain, including trigeminal neuralgia), 14/19 (74%) for phenobarbital (tranquillizer), 13/17 (77%) for allopurinol (asymptomatic hyperuricemia), 11/12 (92%) for oxicams (first line treatment for acute rheumatologic disorders), and 1/3 for phenytoin. These results confirmed that in a majority of cases of SJS or TEN (58%) the prescription of "high risk drugs" could have been avoided. A strict respect of present prescription rules for the main drugs associated with high risk of TEN or SJS could decrease by 30% the incidence of TEN and SJS.

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Adenosine Receptors as Mediators of Both Cell Proliferation and Cell Death of Cultured Human Melanoma Cells

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Adenosine displays contradictory effects on cell growth: it improves cell proliferation, but it may also induce apoptosis and impair cell survival. Following the pharmacological characterisation of adenosine receptor expression on the human melanoma cell line A375, we chose A375 as our cellular model to define how the extracellular adenosine signals are conveyed from each receptor. By using selective adenosine receptor agonists or antagonists, we found that A_{2A} stimulation reduced cell viability and cell clone formation while at the same time it improved cell proliferation. In support of this finding we demonstrate that the stimulation of A_{2A} adenosine receptors stably expressed in CHO cell clone reproduced deleterious effects observed in human melanoma cells. A_3 stimulation counteracted A_{2A}-induced cell death but also reduced cell proliferation. Furthermore, we found that A_3 stimulation ensures cell survival. We demonstrate that adenosine triggers a survival signal via A_3 receptor activation and it kills the cell through A_{2A} receptor inducing a signalling pathway that involves protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs).

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T-Flavanone(t-FN), a Novel Hair Growth Promotor, Suppresses Hair Loss Through Reinforcing Hair Rooting

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This study aims at investigating the mechanism of hair growth promoting activity of t-FN(*trans*-3,4′-dimethyl-3-hydroxyflavanone), a newly synthesized chemical derived from St John's Wort. A double-blind usage test was performed on 84 healthy volunteers who had male pattern baldness. The subjects were divided into three groups with equal average baldness. At the beginning of the test, an evaluation area was set for each subject around the scalp where hair loss was prominent. Each group used, twice a day for six-months, a vasodilator-containing hair lotion supplemented either with 0% (control), 0.1%, or 0.3% t-FN. The efficacy of t-FN was evaluated in the set area, in terms of the total hair number, the terminal hair number, the percentage of terminal hairs, and the hair shaft diameter. At the end of the test period, the terminal hair tenacity was determined as an average peak force required for plucking out a single hair in a nonbald area, with a digital force gauge. In the six-month usage, the terminal hair number, a measure of growing-phase hair ratio, showed a tendency of t-FN dose-dependent increase. This increase was significant in the 0.3% group ($p = 0.02$). The hair tenacity was also increased dose-dependently, being significant in the 0.3% group ($p = 0.03$). This effect could be explained by stiff rooting of the hair in the scalp and/or by mere thickening of the hair shafts. Previously we had demonstrated that t-FN did increase hair shaft diameter. But, interestingly, even when hairs of the same diameter were compared, the tenacity was significantly increased in the 0.3% group ($p = 0.01$). We speculate that t-FN may reinforce the hair rooting independent of the hair thickening. Taken together with our previous *In vitro* studies showing that t-FN enhances the cell adhesion molecule expression in hair follicles, the reinforcement of hair rooting can be a mechanism of the hair growth promotion by t-FN.

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The Influence of Chloroquine Treatment on Minimal Erythemal Dose Measurements in Systemic Lupus Erythemaosus Patients

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Antimalarials have been used in the treatment of systemic lupus erythematosus (SLE) for many years. The mechanism by which they act is not well defined, however, their photoprotective effects are well documented. It was also revealed that IL-6 and IL-18 levels are increased in SLE patients. The aim of our study was to compare minimal erythemal dose (MED) in SLE patients before (BT) and after (AT) prolonged use of chloroquine (CQ) and to investigate whether this treatment may influence the IL-6 and IL-18 sera concentrations in some SLE patients. The study involved 18 patients (17 women and 1 men) aged 24–65 years (mean: 44 y). The diagnosis of SLE was based on the revised criteria of the American College of Rheumatology. Patients included in the study were in inactive phase of the disease, which was determined according to the systemic lupus activity measure (SLAM). Corticosteroids and immunosuppressive drugs were ceased at least 1 month before phototesting and antimalarials at least 6 months. MED measurements were performed typically before and after 3 months treatment with 250 mg of CQ daily. In statistical analysis t-Student, Wilcoxon range and Cochran-Cox tests were used. In 6 patients also IL-6 and IL-18 concentrations in sera were evaluated by ELISA. SLAM score decrease from 9 points (3–15) to 3 (range 1–7) after CQ treatment. MED measurements were as follows: BT: 0.04–0.19 J per cm^2 (mean: 0.10 J per cm^2), AT: 0.07–1.15 J per cm^2 (mean: 0.20 J per cm^2). The differences were highly statistically significant ($p < 0.001$). IL-6 and IL-18 levels was measured in 6 patients and 17 controls. IL-6 concentrations were as follows: BT: mean: 3.5 pg per ml (range 0.8–7.6 pg per ml), AT mean: 2,5 pg per ml (range: 0.9–5.0 pg per ml), Controls: mean: 0.7 pg per ml (0.1–2.0 pg per ml). And in IL-18: 458 pg per ml (205–844 pg per ml), 363 pg per ml (184–530 pg per ml) and 221 pg per ml (72–496 pg per ml), respectively. The differences in IL-6 and IL-18 levels BT and AT were not statistically significant, however, there were statistically significant differences between both samples and the controls ($p < 0.05$). The results showed not only beneficial (SLAM score) but also significant photoprotective effect of chloroquine treatment in SLE patients. Although IL-6 and IL-18 levels decreased during the treatment with chloroquine, the differences were not statistically significant

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The Influence of Chloroquine Treatment on Cholesterol, Triglyceride and Glucose Levels in Systemic Lupus Erythematosus Patients

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Antimalarials have been used in the treatment of systemic lupus erythematosus (SLE) for many years. It was revealed that among other beneficial effects they can reduce cholesterol, trigliceryde and glucose levels, although most studies were performed with hydroxychloroquine (HCQ). As HCQ is not registered in Poland, the aim of the study was to investigate the influence of low doses of chloroquine (CQ) on cholesterol, HDL, LDL, triglyceride and glucose levels in SLE patients. The study involved 16 patients (15 women and 1 men), aged 24–65 years (mean: 45 years). The diagnosis of SLE was based on the revised criteria of the American College of Rheumatology. Patients included in the study were in inactive phase of the disease which was determined according to the systemic lupus activity measure (SLAM < 15) and they did not change their eating habits during the study. Corticosteroids and immunosuppressive drugs were stopped at least 1 month before the study and antimalarials at least 6 months. The blood for laboratory investigations was collected twice: before (BT) and after (AT) 3 months of the daily treatment of 250 mg of CQ. In statistical analysis t-Student test was used. The cholesterol level decreases from 189 mg/% (range: 128–280 mg%) to 167 mg/% (range: 98–233 mg/%) ($p < 0.02$), the trigliceryde level decreases from 147 mg/% (range: 62–267 mg/%) to 98 mg/% (range: 45–199 mg/%) ($p < 0.01$). HDL levels were as follows: BT: 47 mg/% (range: 37–62 mg/%); AT: 45 mg% (range: 25–69 mg/%); ($p = 0.056$) and LDL levels BT: 122 mg/% (range: 71–195 mg/%); AT: 105 mg% (range: 48–158 mg/%); $p = 0.056$. There were no statistically significant differences in glucose levels before and after the treatment. Our results revealed the influence of chloroquine treatment on lipid blood levels in SLE patients. Chloroquine can lower cholesterol and triglicerides levels in SLE patients, which may be beneficial in terms of risk of coronary artery disease especially during corticosteroid treatment.

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Structure-Function Relation of Novel Members of the Efomycine Family

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We have recently described efomycine M, a specific small-molecule selectin inhibitor both *In vitro* and *in vivo*. Since efomycine M is a synthetic derivative of a naturally occuring compound, we were interested in further analysing related molecules and their antiadhesive properties *In vitro*. Fermentation material of *Streptomyces BS 1261* was analysed using FAB-MS spectroscopy and reversed phase-HPLC. Following purification of naturally occurring efomycines, chemical modifications were carried out, and the resulting efomycines were used for cell adhesion assays. The initial analysis of *Streptomyces* fermentation material revealed the presence of 4 major compounds with molecular weights ranging from 1010 to 1052. These compounds were termed efomycines G, E, A and B, representing 14, 68, 16 and 2% of the material, respectively. The most abundant naturally occuring compound, efomycine E, served as the source for modifications resulting in 6 synthetic efomycines: Efomycines N and O are mono-O-methylated and di-O-methylated, efomycines L and M are mono-defucosylated and di-defucosylated. Macrolide-cleavage of efomycine M resulted in efomycine S, and the peracetylated form was termed efomycine T. The potential of these efomycines to inhibit adhesion was tested in experiments assessing neutrophil adhesion to albumin-coated plastic ware, platelets, hypoxia-stimulated porcine aortas, and human umbilical vein endothelial cells (HUVEC). Overall, while the antiadhesive effects of various efomycines were heterogeneous when comparing different matrices, these experiments strongly suggested that efomycines interfered with different selectin-mediated adhesive functions. Thus, we have identified structural motifs necessary for adhesion to certain matrices. Molecular modeling revealed three H-bonding donor/acceptor groups in a defined spatial arrangement which are shared between at least 3 of the efomycines (G, M, T) and the naturally occuring selectin ligand, Sialyl-Lewis^x. This motif is borne on the central macrolide ring system and the rather rigid acyclic side chains. In conclusion, these studies identified small-molecule structural mimetics of selectin ligands which may be particularly amenable as a therapeutic of inflammatory disorders.

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Curcuma longa Extract Combined with UVA or Visible Light Induces Apoptosis in Human Keratinocytes

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Curcuma longa Linn (Zingiberaceae) is a medical plant and spice widely cultivated in tropical regions of Asia and central America. In the form of the herbal powder turmeric, it has been used as an anti-inflammatory remedy in Asian medicine. We could show that Curcuma longa extract (ZCL4) combined with both, UVA or visible light, induces strong growth inhibition in different cell lines. Treatment of cultures of human keratinocytes (HaCaT) with ZCL4 (1–10 μg per ml) plus UVA (1 J per cm^2) inhibited the incorporation rate of BrdU without any detectable release of cytoplasmic lactate dehydrogenase into the supernatant. Using a luciferase reporter assay we found a ZCL4/UVA dependent repression of NF-κB which is thought to be the central regulator of stress responses. Hoechst staining showed an concentration dependent increase of fractionated cell nuclei after treatment with ZCL4/UVA. In addition, the release of cytochrome C from mitochondria (ELISA) corresponded very well with the fragmentation of cell nuclei. Taken together, we suppose the blocking of the NF-κB cell survival pathway and the induction of apoptotic processes after the treatment of human keratinocytes with photo activated ZCL4 without any sign of cell membrane damages up to 10 μg per ml ZCL4. Theses findings could contribute to a new photo therapeutic treatment strategy.

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Idiopathic Angioedema Associated with Estrogen-Containing Oral Contraceptives and Hormonal Replacement Therapy

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Information about recurrent episodes of skin angioedema and abdominal pain attacks caused by oral contraceptives or menopausal hormonal replacement therapy is limited to hereditary angioedema due to C1 inhibitor deficiency type 1 and type 2 and to hereditary angioedema type 3 occurring in females with normal C1 inhibitor. Because of spontaneous reports about an exacerbation of idiopathic angioedema following the intake of oral contraceptives the data of 73 patients were analyzed who had idiopathic angioedema, i.e. recurrent angioedema without urticaria which cannot be classified into the angioedema disease entities known until now. Eight of them were females in whom exogenous estrogens induced angioedema attacks within a latency of 3 days to 3 weeks after starting the medication. In 5 patients clinical symptoms occurred after the intake of oral contraceptives, in 3 patients after starting hormonal replacement therapy. In 5 patients the symptoms occurred for the first time, 3 patients experienced an exacerbation of a previously existent idiopathic angioedema. In the 8 patients C1 inhibitor protein levels, C1 inhibitor function, C4 and C1q concentrations were found to be normal. Various biochemical tests recorded no activation of the fibrinolysis or coagulation system. It is concluded that oral contraceptives or hormonal replacement therapy may cause an induction or exacerbation of nonhereditary idiopathic angioedema.

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In Vitro Diagnosis of Anticonvulsant Hypersensitivity Reaction by Flow Cytometric Assessment of the Cell Cycle

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Anticonvulsants such as carbamazepine or phenytoine may cause the anticonvulsant hypersensitivity syndrome presenting clinically with symptoms including generalised exanthema, Steven Johnson's syndrome, toxic epidermal necrolysis as well as drug induced hepatitis. T-lymphocytes and oxidising enzymes have been shown to contribute to the underlying pathophysiology of this reaction. The polyclonal stimulation of peripheral T-lymphocytes (lymphocyte transformation test, LTT) proved to be helpful in identifying the triggering agent and eventually diagnosing drug allergy (Nyfeler, B., Pichler, WJ., Clin Exp Allergy, 1997) especially when both parent compounds and their metabolites are taken into account (Hertl, M., Jugert, F., Merk, HF., Br J Dermatol., 1995). However, the need for radioactive components to get a read out for this assay (incorporation of ^{3}H-thymidine during DNA-synthesis) prompted us to use nonradioactive read out techniques. Among these determining the amount of drug induced IL-5 via ELISPOT or flow cytometric analysing of cell cycle phases are to note. We improved the conventional LTT by several modifications: PBMC proliferation was determined by a flow cytometric based technique performing DNA staining in order to assess cell cycle phases. Cell cycle S-phase and G_2/M-phase turned out to be reliable read out parameters for PBMC proliferation. This method was compared with both the conventional performed LTT and ELISPOT resulting in similar data with regard to sensitivity and specificity.

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Dimethylfumarate (DMF) Interferes with the Release of Perforin (Perf) Containing Lytic Granules from Cytotoxic T Lymphocytes (CTLs) of Healthy Individuals _In Vitro_

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Systemic therapy of psoriasis with fumaric acid esters is effective. Its molecular mode of action, however, is not known in detail. $CD8^{+}$ cells are major players in the immuno-pathology of psoriasis dominating, e.g. the cellular infiltrate in the lesional epidermis. In addition, as compared to healthy individuals, peripheral blood CTLs of psoriasis patients express more Perf. Since the effect of DMF on $CD8^{+}$ CTLs is not known, we investigated the influence of this drug on the release of Perf-granules from CTLs *In vitro*. Ficoll isolated peripheral blood mononuclear cells of healthy volunteers were preincubated for 1 h with 1, 4, 16, and 40 μmol DMF. Perf-granule release was induced in the presence of DMF with ionomycin and phorbol 12-myristate 13-acetate (PMA). Cells were fixed in 2% formaldehyde after 0, 30, 60, 90, and 120 min, permeabilized with 0.2% saponin, and stained over night with monoclonal antibodies against CD8 and Perf (Hölzel, Cologne, Germany) at 4°C. The percentage of $Perf^{+}$ $CD8^{hi+}$ cells was quantified by flow cytometry. $Perf^{+}$ portion of $CD8^{hi+}$ lymphocytes at time point zero was set for 100%. In all 4 individuals, DMF was found to inhibit Perf-granule release dose-dependently. At 30 min after PMA/ionomycin stimulation, $CD8^{hi+}$ lymphocytes treated with 4 μmol DMF contained a greater portion of $Perf^{+}$ cells (98 ± 14%) as compared to DMF-untreated but PMA/ionomycin-stimulated controls (56 ± 4%; $p < 0.01$). This inhibitory effect was similar at 60 min: 75 ± 11 of initial $Perf^{+}$ lymphocytes treated with 4 μmol DMF still stained $Perf^{+}$ as compared to 52 ± 9% in unstimulated controls ($p < 0.05$). Concentrations of 1, 16, and 40 μmol DMF did not influence significantly Perf-granule release. However, an inhibitory trend was detected with 16 and 40 μmol DMF which might reach significance in larger study groups. Taken together, we report a dose-dependent inhibitory effect of DMF on the PMA/ionomycin-stimulated Perf-granule release from CTLs *In vitro*. This might contribute to the immunomodulatory potential of fumaric acid esters.

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Successful Treatment of Primitive Cutaneous CD30+ T Cell Lymphoma with Topical Imiquimod

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Imiquimod, an imidazoquinoline amine, is an immune reponse modifier drug, which has shown potent antiviral and antitumoral properties in animal models. Although its mechanism of action is little understood, imiquimod stimulates various types of cells to produce interferon alfa, IL12 and other cytokines. On that account it has been initially successfully used topically in genital and perianal warts and newly in the treatment of actinic keratosis, basal cell carcinoma, squamous cell carcinoma, lentigo maligna, cutaneous metastasis of melanoma and Kaposi's sarcoma. Based on these encouraging results and on well-known therapeutic effects of interferon α on cutaneous T cell lymphoma, we decided to treat a 23-year-old male patient suffering from primitive cutaneous CD30+ T cell lymphoma, arising in his right leg three months before our observation. Before starting the treatment the patient was subjected to stadiation, which did not show any involvement other than the skin. The immunohistological esamination disclosed a dermal infiltrate of large lymphocitic cells with CD3+,CD45RO+,CD30+,CD20− phenotype and gene rearrangement of TCRγ demonstrated a monoclonality of T cell population, making the diagnosis of primitive cutaneous anaplastic large cell CD30+ T cell lymphoma. Imiquimod was applied as a 5% cream 3 times per week and 40days after the start of therapy the cutaneous lesion was completely healed: the histological an immunological examination confirmed the total vanishing of T cell infiltrate. The pharmacological properties of this drug, like the capability to stimulate the innate and acquired immunity by the production of numerous cytokines, make it as a novel therapeutic approach to cutaneous lymphoma.

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Comparative Effect of Reference Molecules for Alopecia Treatment in a Murine Model of Hair Growth

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Hair growth stages have been widely studied in C57BL/6 and C3H mice. A feature of these mouse models is that pigment production is exclusively associated to the hair growth so that assessment of skin color allows the monitoring of anagen phase. Our aim was to evaluate the effect of two reference molecules for alopecia treatment in this mouse model. Minoxidil is known to stimulate the vascularization of the skin and finasteride acts as an antiandrogen through the inhibition of 5 α-reductase enzyme. Therefore, minoxidil and finasteride effect was evaluated on hair growth. Anagen phase was induced by wax depilation of the back skin of the two strain mice. Changes in skin pigmentation were assessed by the melanin index meter, chromametry and photographs. Melanin analysis showed that, topically applied once a day for 19 days, 2% minoxidil prolonged the anagen stage by inducing by one day an earlier skin darkening in C57BL/6 mice. Chromametry analysis displayed an effect of minoxidil on C3H mice. Topical (0.6%) but not oral (100 μg per kg) treatment with finasteride once a day for 19 days mice significantly decreased the "a" parameter of chromametry measurement indicating a hair growth. Both molecules seemed not to affect the duration of the cycle: catagen phase appeared at the same moment around day 21 after depilation. Our results showed an efficacy of minoxidil on hair regrowth on the two strains of mice whereas finasteride was only effective in the C3H strain. The C57BL/6 strain can be suited for screening molecules of minoxidil-like activity and the C3H strain, a sensitive strain for oestrogen, can be predictive for assessing molecule exhibiting antiandrogen activity.

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Topical PPAR β/δ and γ Ligands in the Mouse Tail Model and Plaque Psoriasis Assay

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Ligands of nuclear hormone receptors such as glucocorticoid, retinoid, and vitamin D have proved to be candidates in the development of antipsoriatic drugs. PPARs (peroxisome proliferator-activated receptors), which belong to the nuclear hormone receptor superfamily, are pleitropic regulators of immune response, growth, and differentiation in many cell types including the keratinocytes. Several recent reports have dealt with the involvement of PPARα in epidermal processes such as barrier development, proliferation, and differentiation. Little attention has been given however, to the other PPAR subtypes. In the hyperproliferative psoriatic epidermis the expression of both PPARα and PPARγ is decreased, whereas the exact oppposite happens with PPARβ/δ. Ligands of PPARγ include the thiazolidinediones of which oral troglitazone has been shown to be an effective treatment of chronic plaque psoriasis. Pro-differentiating activities and profound antiproliferative action on keratinocytes, as well as critical roles in keratinocyte response to inflammation of PPARβ/δ have been reported. The purpose of our study is to address the effects of tetradecylthioacetic acid (TTA), a potent activator of PPARβ/δ, and rosiglitazone, a specific ligand for PPARγ, both in the mouse tail model in terms of morphological changes, and in clinical trials of therapeutic efficacy on plaque psoriasis. The mouse tail is a relatively sensitive method which allows the evaluation of the effects of antipsoriatic agents on cell differentiation by quantifying the induction of granular layer in the interfollicular regions. Topical treatments of the mouse tail with antipsoriatic drugs such as retinoic acid enhances the conversion of parakeratotic into orthokeratotic cell differentiation (granular cell layer). This model also offers the possibility of measuring the epidermal thickness. The increased antipsoriatic activity is often accompanied with an increase in epidermal thickness, but both reactions are not necessarily related. Epidermal thickness is better regarded as a parameter of skin irritation. The tails of C57BL/6 mice were treated with vehicle (5% tween 80, 10% cetanol, 30% oleo arachidis hydrogenatum, 10% propylenglycol, and 45% aqua conservans), rosiglitazone 0,5%, and TTA 0,5% twice daily over a period of 14 days. Thereafter, longitudinal histological sections were prepared from the tails skin in order to determine the activity on reduction of parakeratosis and epidermal thickness. In the psoriasis plaque assay, eight individuals (5m/3f) suffering from psoriasis were treated twice daily for 30 days with vehicle (n: 14 plaque psoriasis), rosiglitazone 0,5% (n: 18), and TTA 0,5% (n: 18). Assessment of PASI plaque score were carried out at days 0, 4, 7, 11, 14, and 30. In the mouse tail model, neither rosiglitazone nor TTA affected the cell differentiation as no sign of granular layer induction was observed. Although, a slight decrease was demonstrated with TTA (96% ± 3 SEM compared to vehicle), only rosiglitazone is correlated with a statistically significant decrease in epidermal thickness (86% ± 2 SEM compared to vehicle). No significant difference in the reduction of PASI plaque score for total, scale, and infiltration score between the examination times for vehicle, rosiglitazone, and TTA has been observed. For the eythema score, significant differences were observed only at day 7 and 11 between rosiglitazone and TTA. Each treatment was well tolerated by all the patients, with no skin irritation nor adverse drug-related symptoms and dropouts. Although substantial *In vitro* and *in vivo* evidences tend to support the importance of PPARs as critical regulators of disease characterized by hyperproliferation and aberrant differentiation, our clinical observations failed to substantiate the efficacy of rosiglitazone and TTA in an animal model as well in plaque psoriasis assay.

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Pharmacological Study of the Local Effects of a Soluble Oatmeal Extract on Histamine-Induced Inflammatory Reaction in the Skin of Human Healthy Volunteers _In Vivo_

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This study aimed to quantitatively determine the effects of local administration of a soluble oatmeal extract on the development of histamine-induced inflammatory reaction *in vivo*, in six healthy human volunteers. The histamine–induced reaction was quantified during 6 h and at the 24th h in terms of vasopermeability and release of mediators, including neuropeptides (Substance P), proteins, lipid mediator (prostaglandin (PG) E_2) and cytokines (IL-8 et TNFα). The methodology was based on the skin chamber technique: six skin blisters were created on the forearms of the volunteers, epidermal roofs were removed and skin chambers applied on the denuded dermis. One ml of Hanks' solution containing histamine dihydrochloride (1 μM) vs. one ml control Hanks' medium were introduced into two skin chambers. The soluble extract was introduced at four concentrations, 1%, 3%, 10% and 30% (w/v), in 1 μM histamine-containing solution in the remaining chambers. Solutions were renewed hourly during 6 h and left until the 24th h. In the collected samples, protein levels were quantified by Lowry's method, mediators amounts by commercial specific ELISA. The results clearly demonstrated the development of an inflammatory reaction in response to histamine introduction into the chambers, as shown by the significant increase in protein extravasation and mediators during the first hours as compared to control medium. A significant modulatory effect of the oatmeal extract at 1% and 3% was observed on the time-dependent release of substance P according to a dose-dependent manner, with an area under curve of 191.1 ± 233.3 mg per cm^2 and 12.9 ± 22.3 mg per cm^2, respectively, in comparison with control histamine-induced reaction 937.3 ± 455.1 mg per cm^2 (mean ± SD). However, the highest concentration of oatmeal (30% and often 10%) induced some interference with substance P. No major inhibitory effects were observed on proteins, cytokines and lipid mediator. To conclude, the present results show a potent inhibitory effect of oatmeal extract on neurogenic inflammation induced by histamine in healthy human skin.

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Experimental Basis for Beneficial Effects of Pro-/Antioxidants in the Treatment of Viral and Bacterial Skin Lesions

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The leading role of reactive oxygen (ROS) and nitrogen species (RNS) produced by phagocytosing cells in nonspecific antibacterial immunity is well recognized. ROS, mainly hypochlorite and hydroxyl radicals, as well as RNS peroxynitrite are known to kill bacteria extracellularly and help to complete their digestion inside the phagocyte vacuole. The impact of ROS/RNS in viral pathogenesis is unclear as yet. Various viruses, like bacteria, induce defensive ROS/RNS production by both infected cells and by recruited phagocytes, with dual consequence: (1) low intracellular levels of ROS are mitogenic for host cells and promote viral replication, via nuclear transcription factor (NFkB); (2) higher levels of ROS/RNS cause, depending on concentration, either apoptotic or necrotic death of virus bearing cells, thus limiting the spreading of infection. In both bacterial and viral infections, excessive release of ROS/RNS inevitably leads to free radical-mediated inflammatory damage to cells and tissues. Therefore, we suggested that up-regulation of ROS and RNS production by enzyme-inducers and pro-oxidants enhances antibacterial and antiviral immunity; on the other hand, limitation of oxidative damage by antioxidants protects host against cellular death. The pro/antioxidant properties of Immugen® (IDI Farmaceutici, Italy) and its components (ubiquinone, α-tocopherol, L-methionine, selenium aspartate, soy phospholipids) were studied in both *In vitro* and *ex vivo* experiments using methods of electron paramagnetic resonance, chemiluminescence, fluorescent probes, and spectrophotometry that allowed us to distinguish and quantify different ROS/RNS. Results showed that both free radical scavenging and antioxidant properties of ubiquinone and vitamin E were coupled with prominent pro-oxidant and intracellular free radical-generating activity of methionine and soy phospholipids. The former well correlated with production of antiviral cytokines by human leukocytes, their phagocytosing and digestive capacity towards Staphylococcus aureus. Based on these experimental results, a double blind placebo- and case-controlled randomised clinical trial was conducted on 59 patients with recurrent skin warts, proving that cryotherapy and oral administration of Immugen® for 180 days was indeed successful in the treatment of recurrent verruca vulgaris with significant improvement of total viral clearance and lower incidence of relapses.

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Detection of RNase 7 Immunoreactivity in Inflammatory Skin Diseases

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The skin is the largest human organ and constantly exposed to various microbials. Therefore it is surprising that the incidence of clinically manifest skin infections is rather low. There must be potent mechanisms which protect the skin from microbial attacks. In recent studies antimicrobial peptides have been described which are either constitutively expressed by epithelial cells or are induced after contact with infectious agents. One of these peptides is RNase 7, a novel 18–20 kDa cationic protein with antibiotic activity against staphylococcus aureus, pseudomonas aeruginosa, propionibacterium acnes and candida albicans. To obtain antibodies against RNase 7, Balb/c mice were immunized with natural protein purified from psoriatic scales. One out of several monoclonal antibodies raised showed distinct epidermal reactivity on both acetone fixed cryostat sections and formaldehyde fixed, paraffin embedded sections of human skin. In normal human skin, RNase 7 immunoreactivity was found in all epidermal layers. In chronic plaque type psoriasis signals were detected in upper layers below and within parakeratotic layers around microabscesses. Eczema showed a similar pattern whereas in foliculitis a diffuse epidermal staining was found. Surprisingly, dermal vasculature is stained as well. These findings support the idea of an important role of antimicrobial peptides in skin disorders beside protection from infections.

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Vascular Function of Nonlesional Skin in Psoriasis

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The purpose of this study was to investigate the microvasculature status of uninvolved, nonlesional skin of patients with psoriasis. Studies were performed *in vivo* in the skin of the forearm of eight patients with psoriasis and their healthy, age and sex matched controls. Laser Doppler imaging (Moor Instruments Ltd, UK) was used to assess skin blood flux and cutaneous microdialysis to measure tissue levels of NO and total protein using linear 5 kDaA and 3000 kDa molecular mass cut off probes, respectively. The cutaneous vascular response to intradermal injection of substance P (SP, 1×10^{-6} M) and histamine (9×10^{-6} M) was also investigated. Basal blood flux was similar in the nonlesional skin of psoriatics and healthy controls. NO in dialysate from psoriatic nonlesional skin was 47% lower than that recovered from the control group ($0.36 \pm 0.04\,\mu$M and $0.67 \pm 0.04\,\mu$M, respectively, $p = 0.006$). By contrast, basal protein levels were 27% higher in psoriatic skin ($245.5 \pm 6.4\,\mu$g per ml vs. $178.7 \pm 26.4\,\mu$g per ml, $p = 0.02$). The flare response to intradermal SP in nonlesional psoriatic skin ($8.4 \pm 1.4\,cm^2$) was significantly lower than that in controls. ($13.8 \pm 1.9\,cm^2$) ($p = 0.04$) as was itch. There was no signifiacnt difference in the histamine-induced flare or itch. Together, these data suggest that microvascular function is altered in nonlesional skin of patients with psoriasis and that the cutaneous microvascular abnormalities seen in psoriasis are not confined to psoriatic plaques.

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Clonality Detection in Cutaneous B-Cell Lymphomas Using Immunoglobulin Heavy Chain Gene Rearrangement PCR and Fluorescence Fragment Analysis on Automated DNA Sequencer

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Detection of clonally expanded immunoglobulin heavy chain (Igh) gene rearrangements by PCR and subsequent electrophoresis is increasingly used in the diagnosis of cutaneous B-cell lymphomas (CBCL). To this end, primers for the three Igh framework regions (FR1,2,3), the leader sequence and the Jh region are applied, all amplifying the highly variable Igh third complementary region (CDR3). Recently, fluorescence PCR-fragment analysis on automated DNA sequencers (GeneScan analysis, GSA), providing an exact sizing has been applied as appropriate separation technique in this context. We have evaluated all Igh primers hitherto known by a PC primer analysis program. Then, fluorescently labeled products generated from DNAs of 58 paraffin embedded and 5 frozen lesional skin biopsies of confirmed CBCL cases, using the primer sets selected, were analyzed by GSA on the ABI 310 Prism instrument. Single round or seminested FR3/Jh-PCR showed clonal B-cells only in 32.6 or 37% of cases, respectively. This fraction was increased to 54.3% including a nested FR1/Jh-PCR, and, to 60% applying a supplementary nested FR2/Jh-PCR. However, false clonal results, indicated by peaks of varying sizes from repeated PCR, have been received by nested PCR, mostly by FR2/Jh. Obviously due to their poor quality, the Igh leader-PCR has not yielded amplification products from paraffin-derived DNAs. Our data show that the FR3/Jh-PCR only is not sufficient for detecting B-cell clonality in CBCL, but following inclusion of additional Igh-PCR, a substantial number of cases still fail to show clonality.

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Histopathological and Ultrastructural Changes in Human Skin as a Result of Black Fly Attack in Suburban Area of Warsaw, Poland

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Black flies, cosmopolitan cattle ectoparasites from genus *Simulium*, are insects of great medical importance because of their massive attack on humans; they represent vectors of onchocercosis and probably anthrax. The aim of this study was to assess cutaneous changes in man caused by a severe invasion of black flies (*Simulium* sp.). Twelve men and women (19–62 years old) with the dermal disorders caused by black fly bites were assessed clinically. Skin biopsy for the histopathological and the transmission electron microscope examinations was taken from the man with the most serious symptoms. All persons showed changes of various intensity situated mainly on lower limb skin. Some of them had a local allergic reaction maintaining for up to two weeks. Small painful petechial spots, swellings and pruritic blisters, and later greater erythematous changes around a few sites of bites occurred. Blisters were bleeding profusely during a long time after scratching. The clinical assessment showed that the cutaneous changes were healing fully when the antihistamine and antibiotic treatments have been applied during seven weeks after the black fly invasion. The histopathological examinations revealed the skin lesions, damages of the blood capillaries and inflammatory process developing in sites of black fly bites. Infiltration consisted of different cells and the oedema with extravasated blood occurred. Clear changes inside and outside the dermal cells were found by means of TEM in skin biopsy material. Numerous macrophages filled with phagolysosomes occurred in the extracellular dermal space. Conglomerations of the mast cells were also found. Ultrastructure of the analysed material indicated advanced process of rebuilding in the dermal cell region developing with the intensive neoangiogenesis. The above described skin changes are connected with various adaptations of the mouthpart that enable flies to feed the blood. Contents of fly saliva and the toothed bladelike cutting mouthpart cause bite wounds with damages of dermal cells and blood capillaries. Different personal susceptibility may influence various intensiveness these changes. Better knowledge of skin lesion types, caused by *Simulium* may be useful for a proper diagnosis, treatment and for preventing of serious secondary infections.

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Withdrawn

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Skin Mapping Using Low Cost Stereo Vision

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In dermatology assessment of localization and extent of skin involvement is often a prerequisite for therapy planning and evaluation of therapeutic effects. So far, this has largely been done by subjective, semiquantitative scoring systems. Digital images, in contrast, can provide an objective and comprehensive database, but they contain only two-dimensional information and are therefore inappropriate when large areas of the body surface have to be considered. The reason for this are the many occlusions and distortions introduced with a simple 2D imaging approach. Therefore three-dimensional information has to be obtained so that a geometric rectification of skin surface patches allows quantitative measurements. We propose two off-the-shelf digital cameras to acquire a stereo data set from the skin surface. A first image pair with random texture projected is used to generate 3D information. One colour image of the skin texture completes the input data set for stereo reconstruction. Dense stereo matching is performed, then the resulting point cloud is overlaid with texture from the skin surface. This allows the expert to work on a geometrically correct high-resolution virtual model of the patient's surface. Recognition of lesional skin is further based on colour and texture analysis. A prototype system yielding two oblique views from the back of the trunk was used to evaluate patients with multiple melanocytic lesions and patients with plaque-type psoriasis. In using a texture based matching method designed to obtain a dense grid of correspondences on fairly smooth surfaces (such as most patches on the human skin) stereo reconstruction produces a three-dimensional view of the skin surface. Lesional skin was automatically detected by means of tissue counter analysis, yielding quantitative data as to the extent of skin involvement and as to the localization of individual lesions. The skin mapping prototype described in this study is a first step towards digital mapping of the entire skin. The final goal is a skin mapping equipment for follow-up examinations in pigmented lesion screening as well as for an objective quantitative assessment of therapeutic effects in inflammatory dermatoses.

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Pseudopelade of Brocq: Immunohistochemical Definition of Different Infiltrating Patterns

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Twelve cases of clinically diagnosed Pseudopelade of Brocq (PB) were studied by immunohistochemistry to evaluate antigenic features of the infiltrate and the presence of fibrogenic and antifibrogenic cytokines. On the basis of both histology and direct immunofluorescence, two cases could be diagnosed as discoid lupus erythematosus (DLE), and 3 cases as lichen planopilaris (LPP). The other 7 cases were classified as "non characterized pseudopelade" (NCPP). By immunohistochemistry the pattern of the infiltrate was assessed. Two major patterns were defined: (1) conspicuous periadnexal/interstitial infiltrate of CD3+ cells with high CD4+/CD8+ ratio and a variable amount (always less than infiltrating lymphocytes) of macrophages, mastocytes, and fibroblasts; (2) unconspicuous periadnexal/interstitial infiltrate of CD3+ cells with variable CD4+/CD8+ ratio, and conspicuous amounts of macrophages, mastocytes and fibroblasts in various proportions. The first pattern was typical of the cases classified as DLE or LPP, the second pattern was representative of the cases classified as NCPP. IL-4 was expressed by lymphocitic infiltrate nearly in all cases, IL-6 was observed on lymphocytes and macrophages in all cases, IFN-γ was found in LP infiltrating lymphocytes, bFGF was always expressed on macrophages. Immunohistochemistry of our PB cases allowed us to identify two different infiltrating patterns and to recognize the consistent presence of fibrogenic cytokines.

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Cd44 Expression in Mouse Epidermis After Topical Application of Retinoids and UVB Irradiation

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CD44 is a polymorphic transmembrane glycoprotein and the principal cell surface receptor of hyaluronate (HA). Recently we have shown that the topical application of retinaldehyde (RAL) resulted in an epidermal hyperplasia and a marked increase of CD44 expression in the follicular and interfollicular epidermis in C57BL/6 and SKH1 hairless mice. In another study, we also showed that CD44 expression was significantly reduced in the membrane and became cytoplasmic 2 h after UVA (10 j per cm^2) or UVB (1 j per cm^2) and reconstituted within 8 or 24 h for UVA and UVB, respectively. In a previous study, we demonstrated that topical application of 0.05% RAL for 3 days prevented the decrease of CD44 and HA in the epidermis induced by UVA and UVB irradiation. In the present study, we aimed to explore the expression of CD44 in the epidermis of the back skin of SKH1 hairless mice after UVB irradiation (1 j per cm^2) which was previously treated with 0.05% retinaldehyde (RAL), 0.05% retinoic acid (RA), and 0.05% retinol (ROL) topically for 7 days. For this purpose, an immunohistochemical study was performed on the skin sections of mice by using biotinylated anti-CD44 antibodies. Our results showed that all the retinoids increased the expression of epidermal CD44. The CD44 expression was more strongly increased in mice treated with RAL when compared to those treated with RA and ROL. In addition, RAL prevented the decrease of CD44 induced by UVB irradiation more significantly when compared to RA and ROL.

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Association of hsp27 with Epidermal Structural Proteins and its Expression in Keratinization Disorders

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Hsp27 is a small heat shock protein, which is expressed in an ubiquitous and intracellular way. This stress protein has been highly conserved during evolution. In human epidermis its expression is associated with keratinocyte differentiation. Hsp27 functions as a molecular chaperone by binding to other proteins, e.g. actin. Concerning the differentiation associated expression pattern of hsp27 the aim of this study was to provide evidence for a possible function of hsp27 as a molecular chaperone in human keratincytes especially during the assembly of the CCE. We investigated the expression- and colocalization pattern of hsp27 and filaggrin, loricrin, transglutaminase 1, keratins, and actin in normal human skin. Evaluation was done by confocal laser scan microscopy and electron microscopy. Furthermore we investigated a panel of kerinization disorders for the expression of hsp27 by immunohistochemistry. In more than 60% of the samples we could detect subcorneal colocalization of hsp27 with loricrin, filaggrin, transglutaminase 1, and keratins. Colocalization with actin could not be revealed. For loricrin and filaggrin we could show that ultrastructurally the obtained colocalizations are caused by binding of these antigens to tonofilaments in close vicinity. Most of the keratinization disorders we investigated expressed hsp27 in a regular pattern corresponding to normal skin. However, 3 out of 3 patients with chondrodysplasia punctata (CDPX) lacked epidermal expression of hsp27. From these results we conclude that hsp27 might serve as a molecular chaperone of tonofilaments and of the cornified cell envelope. Further studies will address the role of the lack of hsp27 in the pathophysiology of CDPX.

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Pityriasis Lichenoides is a Polyclonal T Cell Disorder with a Variable Recruitment of the Different Subpopulations of CD8+ Cytotoxic T-Cells along its Various Histological Phases

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Pityriasis lichenoides (PL) chronica and its acute form, pityriasis lichenoides et varioliformis acuta, are skin diseases of unknown origin that probably represent an hypersensitivity reaction to an infective agent. PL is a benign disorder but some authors, for the detection of monoclonal T-cell populations both in the chronic and acute form, have suggested that PL might belong to the group of primary CTCL. Other authors showed that the inflammatory infiltrate in PL is composed by CD8+ T-cells but they did not detect cytotoxic molecules. These data prompted us to evaluate in 60 cases of PL: (a) the T-cell receptor (TCR) g gene rearrangement; (b) cytotoxic proteins expression like TIA-1 and Granzyme-B (Gr-B); (c) the apoptotic index by means of "Tunel" analysis and detection of activated caspase-3; (d) the phenotypical profile, by testing serial tissue sections with monoclonal and polyclonal sera against CD2, CD3, CD4, CD8, CD45RA, CD45RO, CD56. All the studies have been performed on formalin-fixed and paraffin-embedded materials. The cases analysed were further subdivided, according to specific histological parameters, into early, fully developed and late lesions. Our results demonstrate that the infiltrate in PL early lesions is composed by CD2+, CD3+, CD8+, CD56–, T-cells, whereas CD8+ cytotoxic T-cells with a phenotype indicative of the naïve (CD45RA+, CD8+, CD56–, TIA-1+), memory (CD45RO+, CD8+, CD56–, TIA-1+) and effector populations (CD45RA+, CD8+, CD56–, TIA-1+, Gr-B+) are quite exclusively observed in fully developed and late lesions. Gr-B is variably expressed from case to case but both with a clear correlation with histological signs of acuteness and of cytopathic damage, the more the signs the more the percentage of Gr-B+ cells, and with the apoptotic index. Finally, TCR g gene rearrangement analysis gave a polyclonal signal in almost all the analysed cases. In conclusion, data obtained so far suggest that PL is a polyclonal T-cell disorder linked to CD8+ cytotoxic T-cell activity.

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CD40-CD154 Ligation Between Keratinocytes and T Cells in Psoriatic Lesion and Keratinocyte Production of Chemokines by This Ligation *In Vitro*

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In psoriatic lesions, activated T cells and CD40$^+$ keratinocytes are seen. We argued that some of the activated T cells may be CD154$^+$ cells which may ligate with CD40$^+$ keratinocytes. This ligation may induce the release of chemokines and complement by keratinocytes and alter their expression of complement (C) regulatory proteins; abnormalities seen in psoriatic lesion. To test this, we examined CD40-CD154 ligation in the lesion of 10 patients with psoriasis *in situ*. We also studied, *In vitro*, the keratinocyte release of chemokines (IL-8, RANTES and MCP-1) and C components (C3 and factor B) by ELISA and alteration of expression of C regulatory proteins (DAF, MCP and CD59) by flow cytometry in response to this ligation. For CD40-CD154 ligation on keratinocytes, we first treated keratinocytes with IFN-(in culture to up-regulate CD40 expression and then incubated them with CD154 transfected J558 cells or soluble CD154. In the lesion, CD40$^+$ keratinocytes were seen in all patients; in relatively larger clusters in stratum spinosum, smaller clusters in stratum basale and occasionally in small clusters in stratum granulosum. CD154$^+$ T cells were seen exclusively in stratum spinosum in seven patients; in six in juxtaposition to CD40$^+$ keratinocytes. These results indicated CD40-CD154 ligation between keratinocytes and T-cells in the lesion. *In vitro*, keratinocytes produced small amounts of IL-8, RANTES and MCP-1 in culture medium but CD40-CD154 ligation on them greatly enhanced the production of these chemokines (10-, 10-, and 3-fold, respectively) without inducing the production of C3 and factor B and without altering the expression of MCP, DAF or CD59. Up-regulation was time and dose dependent. Contact between CD154 transfected cell and CD40$^+$ keratinocytes was essential for up-regulation of chemokine production as seen in a Transwell system. An anti-CD40 monoclonal antibody almost completely blocked IL-8 induction in response to this ligation. Interaction between CD154$^+$ T cells and CD40$^+$ keratinocytes in the epidermis of psoriasis patients may play a role in the genesis of a local inflammatory process via chemokine production by keratinocytes.

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Morphology of Sweat Glands and Periglandular Nerves in Hyperhidrotic Palms Before and After Intradermal Botulinum Toxin

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Intradermal botulinum toxin (Btx) produces long-lasting relief of focal hyperhidrosis. Although Btx blocks cholinergic transmission, its mechanism of action in hyperhidrosis is poorly understood. Objective: To study the effect of Btx A on the size and innervation of sweat glands in patients with palmar hyperhidrosis.
Palmar skin biopsy specimens from 20 hyperhidrotic patients and 11 controls were investigated. Nine of the patients also underwent biopsy 2.5-6 months after Btx injections when no relapse of sweating had yet occurred. Sweat gland morphology (diameters of the secretory tubule and tubular lumen) was investigated by light microscopy after routine haematoxylin staining. Immunofluorescence with antibodies to the neural marker protein gene product 9.5 (PGP 9.5) and to vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) was used to analyse the periglandular innervation. Results: Tubular dimensions in patients with hyperhidrosis after Btx treatment were similar to those in control subjects. The tubular lumen had diminished ($p<0.05$) and a tendency to decreased PGP 9.5-like immunoreactivity ($p=0.09$) around the glands was seen. No change in VIP or CGRP immunoreactivity was observed post-treatment.
Both the periglandular innervation and the sweat gland per se seem to be affected by intradermal botulinum toxin.

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Morphometric Diagnosis of Histologic Sections of Melanocytic Skin Tumors in Automated Image Analysis

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In tissue counter analysis, digital images of tissue scenes are dissected into elements of equal size and shape, and the contents of each element are evaluated by a set of grey level, color and texture features. The aim of this study was to test the applicability of tissue counter analysis and CART (Classification and Regression Tree) to the diagnostic discrimination of a large series of histologic sections of benign common nevi and malignant melanoma. Two hundred cases each of benign nevi and malignant melanoma were consecutively sampled. After creation of datasets based on 10 cases each, CART analyses of background vs. tissue elements and cellular vs. other tissue elements were performed. In a second step, a learning set of 120000 cellular elements obtained from 100 cases each was created. Based on the learning set, CART analysis was performed in order to differentiate between benign and malignant cellular elements. For diagnostic assessment, only the percentage of cellular elements suggestive for malignancy in each case was used. In the learning sets, CART analysis led to a correct classification of 99% of background vs. tissue elements, 96% of cellular vs. other tissue elements and 79.1% of benign vs. malignant cellular elements. When the percentage of cellular elements suggestive for malignancy in each case was evaluated it turned out that a threshold level of 52.51% provides a correct classification of 192 nevi and 186 melanoma out of 200 each (specificity 96%, sensitivity 93%, positive predictive value 95.9%). An overall performance of 94.5% correctly classified cases in a total of 400 histologic sections of melanocytic skin tumors clearly suggests that tissue counter analysis combined with CART (Classification and Regression Tree) is a powerful tool for diagnostic purposes in histopathology.

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CD44 and Hyaluronate Expression in Follicular Mucinosis

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CD44 is a membrane glycoprotein and serves as the major cell surface receptor for hyaluronate (HA). In a recent study we have demonstrated that the lack of CD44 expression in murine keratinocytes leads to an abnormal HA accumulation in the superficial dermis, indicating an important role of CD44 in local HA metabolism in the mouse skin. We have also observed a decrease in the expression of epidermal CD44 in patients of lichen sclerosus et atrophicus which is potentially responsable for the dermal deposition of HA in this disease. Recently we have also shown that HA accumulation was associated with decreased expression of CD44 in trichofolliculoma-like epithelial proliferations in perifollicular solitary cutaneous myxoma. In this study we examined the follicular expression of CD44 and HA in the skin biopsy specimens of seven patients with follicular mucinosis by using CD44-specific monoclonal antibodies and biotinylated HA-binding protein (HABP), respectively. No difference of CD44 expression was observed in the follicular keratinocytes when compared with those of unaffected interfollicular epidermis. The follicular zones of mucin deposition were strongly positive for HA. A weak interkeratinocyte staining pattern for HA was also observed in the interfollicular epidermis. However, HABP staining revealed a stronger reactivity in the follicular keratinocytes surrounding the mucin-accumulated areas compared to the interfollicular keratinocytes, indicating an active secretion of HA by follicle cells in follicular mucinosis.

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Amyloid Elastosis: A Rare Variant of Al Systemic Amyloidosis

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A 56-year-old woman presented with polyneuropathy and yellowish coalescing macules in intertriginous areas resembling pseudoxanthoma elasticum (PXE) or diffuse plane xanthomas. Two lesional skin biopsies were obtained from the axilla and the thigh. Both specimens demonstrated small globules of slightly basophilic amorphous material between collagen bundles throughout the dermis. These deposits were PAS-positive and showed fluorescence with Thioflavine-T. They strongly stained with Congo red and showed the characteristic green birefringence of amyloid substance when viewed under polarized light. Orcein stain revealed that amyloid was preferentially deposited on elastic fibers that appeared to be coated with a thick layer of unstained amyloid substance. Immunostaining using monoclonal mouse antibody to amyloid A remained negative. Electron microscopic studies confirmed that the microfibrillar amyloid substance was closely associated with elastic fibers in the dermis. Amyloid was further detected in muscle, nerve, gastric, duodenal and bone marrow biopsy specimens. The patient had medullary plasmocytosis with lambda light chain restricted expression. She underwent autologous stem cell transplantation, which resulted in progressive regression of cutaneous signs and stabilisation of the polyneuropathy. Amyloid elastosis is a rare variant of systemic amyloidosis characterized by prominent amyloid deposition on elastic fibers, in close association with their microfibrils. Only 2 cases of amyloid elastosis with PXE-like features and a rapid fatal outcome have been previously described.

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Withdrawn

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Penile Lichen Sclerosus and Carcinoma

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The relationship between penile lichen sclerosus (LS) and cancer development has not been clearly assessed so far. In order to define those histological features of LS that may indicate or precede a malignant degeneration, 104 biopsy specimens from 86 patients with LS of the glans and from 9 patients with a penile malignancy arising on LS were reviewed. Three different histopathologic LS patterns were identified – a first pattern with a prominent lichenoid inflammatory infiltrate in the dermis (9%), a second pattern characterized by a band-like infiltrate separated from the epidermis by a band of dermal sclerosis (44%), and a third pattern showing prominent sclerosis with minimal or absent inflammatory infiltrate (9%). In 38% of cases we also found a fourth pattern, with overlapping features between the first and the third pattern, occasionally showing areas of epidermal thickening, with loss of the normal keratinocyte cytoarchitectural differentiation, mitoses and apoptotic cells. The histological features observed in the fourth pattern may be interpreted as areas of disease reactivation within a chronic stage. Furthermore, 7 out of 9 cases of penile cancer from our series (78%) were associated with this pattern of LS, suggesting that it may correlate with a malignant degeneration.

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Ultrastructural and Genetic Studies in Patients with Autosomal Recessive Congenital Ichthyoses

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Skin ultrastructure and TGM1 mutations were studied in seven families with autosomal recessive congenital ichthyosis (ARCI). Two patients with lamellar ichthyosis (LI) showed similar rectangular clefts within the corneocytes (IC typeII). One of them was heterozygote for known missense mutations (A3366G/V378L), the other one had a novel amino acid substitution R553P within the TGM1 gene. Five patients fulfilled clinical criteria of congenital ichthyosiform erythroderma (CIE), one of them, however, had had erythroderma only in childhood. We found rectangular clefts in the horny cells in one CIE patient, who proved to have a V378L change on one allele in TGM1. Here we diagnosed a CIE-like LI, possibly initiated by retinoid treatment. Skin ultrastructure of the second patient detected thinner cornified cell envelopes and lipid droplets in the corneocytes (IC type I). The patient was heterozygote for two novel TGM1 nonsense mutations Y503X/S669X, resulting in a truncated TGase 1 molecule. The third patient, who presented with mild scaling without erythema, was classified as CIE because of a continous erythroderma during his early childhood. Skin ultrastructure proved perinuclear membranous structures in the granular and membranous bundles within the horny cells (IC III). No underlying TGM1 mutations had been verified. Ultrastructural features of the remaining two patients displayed two unique features. They had no keratohyalin granules but had multiple vacuoles filled with electrondense granular material within the granular and horny cells. Neither TGM1 gene mutations, nor hairshaft abnormalities could have been detected.

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Witdrawn

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Giant Cell Lichenoid Dermatitis in a Patient with Baboon Syndrome

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Giant cell lichenoid dermatitis is a recently described pathological entity which can be seen as an unusual lichenoid drug eruption, a manifestation of sarcoidosis or within herpes zoster scars. Histopathological findings include focal vacuolar alteration of the basal layer with cytoid bodies, dermal and intraepidermal multinucleated giant cells, and a mixed chronic inflammatory infiltrate with a lichenoid pattern consisting of lymphocytes, histiocytes, eosinophils and plasma cells. Here we report a giant cell lichenoid dermatitis in a 41-year-old male patient who developed, 3 days after intravenous treatment with amoxicillin-clavulanic acid for erysipelas of the left leg, a clinical picture suggesting a Baboon syndrome characterized by an erythematous and purpuric eruption on the axillary, inguinal and popliteal areas and the anterior side of elbows. Histologically there was an epidermal hyperplasia and atrophy with focal vacuolar alteration of the basal layer, exocytosis and cytoid body formation. A band-like lichenoid inflammatory infiltrate containing lymphocytes, histiocytes, eosinophils, plasma cells and epidermotropic CD68+ multinucleate giant cells was found at the dermoepidermal junction and in the superficial dermis. This is the first reported case of giant cell lichenoid dermatitis in a patient with Baboon syndrome.

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Vasoactive Intestinal Polypeptide Receptor $VPAC_2$: Localisation of mRNA-Expression in Human Skin

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The bioactive peptide vasoactive intestinal polypeptide (VIP) is involved in the regulation of various aspects of cutaneous cell differentiation. VIP is abundantly present in cutaneous autonomic nerve fibers where it acts as neuromodulator and participates in the regulation of regional blood flow and pain transmission. We studied the distribution of the inducible receptor subtype $VPAC_2$ mRNA in the human skin using a human specific $VPAC_2$ cRNA probe. Non-isotopic in-situ-hybridisation for the receptor mRNA was simultaneously combined with immunohistochemistry for the ligand VIP. $VPAC_2$ mRNA hybridisation signals were detected in a variety of cutaneous cell types. There was marked staining of epidermal cells with most pronounced hybridisation signals found in keratinocytes of the basal layer. In deeper regions, glandular cells surrounded by VIP-immunoreactive nerve fibers were positive for $VPAC_2$ mRNA. Hair follicle cells next to VIP-positive fibers also exhibited hybridisation signals. Specific staining was also detected in mononuclear cells. No signals were obtained in vascular smooth muscle myocytes and endothelium and connective tissue. There was no difference in the pattern of distribution between biopsies of different areas. In summary, $VPAC_2$ mRNA is localised in keratinocytes, glandular, hair follicle and immune cells of the skin but not in vascular smooth muscle indicating that the effects of VIP on vasodilation may be mediated via a different receptor or by paracrine pathways.