Abstracts for the 26th Annual Meeting of the European Society for Dermatological Research

Amsterdam, September 28–October 1, 1996

ESDR MEETING AMSTERDAM 1996

Saturday, September 28, 1996

From Basic Research to Better Care—A clinically oriented joint symposium of the ESDR and The Netherlands Society for Dermatology and Venereology which celebrates its 100th Anniversary in 1996:

10:30–10:35	Welcome and Introduction
10:35–11:10	Epidemiology of atopic dermatitis Dr. T. Diepgen, Erlangen
11:10–11:45	Atopic dermatitis: new developments Prof. Dr. C. Bruijnzeel-Koomen, Utrecht
11:45–12:30	Guest lecture: Epidermal cytokines, adhesion molecules and skin disease Prof. Dr. T. Kupper, Boston, U.S.A.
12:30–13:45	*Lunch*
13:45–14:20	A molecular genetic approach to the analysis of primary determinants for psoriasis Dr. Richard Trembath, Leicester
14:20–14:55	Development of a new engineered human monoclonal antibody for psoriasis therapy Dr. Martyn Robinson, Celltech Ltd, U.K.
14:55–15:30	New developments in psoriasis therapy Prof. Dr. T. Ruzicka, Düsseldorf
15:30–16:00	*Coffee*
16:00–16:35	The molecular dissection of junctional epidermolysis bullosa Prof. Dr. J-P. Ortonne, Nice
16:35–17:10	Advances of clinical importance in photobiology Prof. Dr. J. Krutmann, Düsseldorf
17:15–18:00	***RUDI CORMANE LECTURE*** ***Immunodermatology in the molecular era: novel tools and new challenges*** ***Prof. Dr. G. Stingl, Vienna***
18:00–18:30	Anniversary celebrations
18:30–20:30	Welcome reception courtesy of the Netherlands Society for Dermatology and Venereology

Sunday, September 29, 1996

08:15–10:15	***CONCURRENT MINISYMPOSIUM*** ***Keratinocyte biology (Chairpersons: I. Leigh, London; M. Ponec, Leiden)***
08:15–08:40	Y. Barrandon, Paris: What is a keratinocyte stem cell?
08:40–09:05	N. Fusenig, Heidelberg: Epithelial-mesenchymal interactions regulating keratinocyte growth and differentiation
09:05–09:30	B. Lane, Dundee: Keratinocyte differentiation and structural proteins
09:30–09:55	D. Hohl, Lausanne: Cornification—the roles of transglutaminase and the cornified cell envelope
09:55–10:15	General discussion and posters
08:15–10:15	***CONCURRENT MINISYMPOSIUM: Dermato-epidemiology meets the basic sciences (Chairpersons: H. Williams, Nottingham; J. Bavinck, Leiden; organised by the European Dermato-Epidemiology Network)***
08:15–08:35	H. Williams, Nottingham: Why should dermato-epidemiology concern basic scientists?
08:35–08:55	J-C Roujeau, Crêteil: Mechanisms and causes of severe cutaneous drug reactions: improved knowledge from epidemiology
08:55–09:15	J. Bavinck, Leiden: Skin carcinogenesis in transplant patients
09:15–09:55	Poster discussion
09:55–10:15	L. Naldi, Bergamo: Smoking and psoriasis—can biology and epidemiology meet?
10:15–10:45	*Coffee*
10:45–13:15	***NON-CONCURRENT MINISYMPOSIUM: Melanoma (Chairpersons: R. MacKie, Glasgow; B. Vermeer, Leiden)***
10:45–11:30	Guest lecture by Dr. P.I. Schrier, Leiden: Melanoma antigens. Targets for vaccination?
11:30–11:50	N. Gruis, Leiden: Molecular genetics
11:50–12:10	M. Edwards, Glasgow: Cell adhesion molecules, integrins and metastatic potential
12:10–12:30	J. Johnson, Munich: Melanoma associated genes
12:30–12:50	T. Luger, Münster: Cytokines in melanoma
12:50–13:15	Roundtable discussion: R. MacKie, Glasgow; B. Vermeer, Leiden; A. Thody, Newcastle; D. Norris, Denver
13:15–14:00	*Lunch*
14:00–16:00	Poster viewing
16:00–18:15	***NON-CONCURRENT MINISYMPOSIUM: Psoriasis (Chairpersons: P. van de Kerkhof, Nijmegen; Th. Ruzicka, Düsseldorf)***
16:00–16:02	Th. Ruzicka, Düsseldorf: Introduction
16:02–16:24	J. Barker, London: Cytokine network
16:24–16:46	F. Nestle, Zürich: Antigen presenting cells and T cells
16:46–17:08	G. Michel, Düsseldorf: Immunomodulation as an antipsoriatic principle
17:08–17:30	P. van Erp, Nijmegen: Epidermal growth
17:30–17:52	I. Leigh, London: Epidermal differentiation
17:52–18:14	C. Carlberg, Geneva: Molecular mechanisms of the antiproliferative effect of vitamin D_3 and its analogues
18:14–18:15	P. van de Kerkhof, Nijmegen: Concluding remarks
18:30–19:30	**Presidential address and Annual General Meeting**

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Monday, September 30, 1996

08:15–10:15 ***CONCURRENT MINISYMPOSIUM: Atopic dermatitis (Chairpersons: J. Bos, Amsterdam; K. Thestrup-Pedersen, Aarhus)***

08:15–08:20 K. Thestrup-Pedersen, Aarhus: Introduction
08:20–08:35 T. Bieber, Munich: Dendritic cells and atopic dermatitis
08:35–08:50 M. Kapsenberg, Amsterdam: Cytokine regulation in Th cells
08:50–09:05 C. Neumann, Göttingen: Cytokine profiles of T cell clones from skin biopsies
09:05–09:20 J. Krutmann, Düsseldorf: *In situ* determination of T-lymphocyte-derived cytokine expression
09:20–09:35 O. Baadsgaard, Copenhagen: Superantigens and atopic dermatitis
09:35–09:50 K. Thestrup-Pedersen, Aarhus: Non-antigen but cytokine-driven proliferation of skin-homing T lymphocytes in atopic dermatitis
09:50–10:10 P. Friedmann, Liverpool: Type I and type IV cutaneous reactions and the effect of allergen avoidance in atopic dermatitis
10:10–10:15 J. Bos, Amsterdam: The next 25 years

08:15–10:15 ***CONCURRENT MINISYMPOSIUM: Oncogenes and cell cycle control (Chairpersons: N. Basset-Séguin, Montpellier; J. Rees, Newcastle)***

08:15–08:20 N. Basset-Séguin, Montepellier: Introduction
08:20–08:40 M. Heenen, Brussels: cell cycle control and carcinogenesis
08:40–09:00 M. Ueda, Kobe: Ultraviolet, oncogenes and the cell cycle
09:00–09:20 N. Gruis, Leiden: The role of P16
09:20–09:40 A. Quinn, London: Genetics of basal cell carcinoma and Gorlin's syndrome
09:40–09:55 J. Molés, Montpellier: The P53 gene in skin carcinogenesis
09:55–10:10 E. Healy, Newcastle: Melanoma, genetic change and MSH variations
10:10–10:15 J. Rees, Newcastle: Conclusion

10:15–10:45 *Coffee*

10:45–13:00 ***NON-CONCURRENT INTERNATIONAL MINISYMPOSIUM: Cytokine biology (Chairpersons: K. Takehara, Kanazawa; E. O'Keefe, Chapel Hill; T. Luger, Münster)***

10:45–11:20 Guest lecture by Prof. T. Kupper, Boston: Can a transgenic approach to the analysis of skin disease show us the way?
11:20–11:45 M. Cork, Sheffield: Cytokine gene polymorphisms in inflammatory dermatoses
11:45–12:10 A. Hatamochi, Chiba: Role of cytokines in controlling connective tissue gene expression
12:10–12:35 K. Takehara, Kanazawa; A. Igarashi, Kanto Teisin: Connective tissue growth factor (CTGF) and skin fibrotic disorders
12:35–13:00 T. Schwarz, Münster: The role of interleukin 12 in cutaneous biology

13:00–14:00 *Lunch*

14:00–16:00 Poster viewing

16:00–18:00 ***CONCURRENT MINISYMPOSIUM: Genodermatosis; Recent developments (Chairpersons: L. Bruckner-Tuderman, Münster; R. Eady, London)***

16:00–16:05 L. Bruckner-Tuderman: Introduction
16:05–16:20 G. Meneguzzi, Nice: Laminin 5 and $\beta 4$ integrin mutations in junctional epidermolysis bullosa
16:20–16:35 J. McGrath, London: Bullous pemphigoid antigen 2 (BPAg2, BP180) abnormalities in generalized atrophic benign epidermolysis bullosa
16:35–16:50 M. Jonkman, Groningen: Newly characterized forms of epidermolysis bullosa simplex
16:50–17:05 I. McLean, Dundee: Epithelial fragility disorders resulting from mutations in differentiation-specific keratins
17:05–17:20 D. Hohl, Lausanne: Molecular genetics of lamellar ichthyosis
17:20–17:35 M. Pope, Cambridge: Heterogeneity of Ehlers-Danlos syndrome
17:35–17:55 General discussion and posters
17:55–18:00 R. Eady, London: Conclusion

16:00–18:00 ***CONCURRENT MINISYMPOSIUM: Adhesion molecules (Chairpersons: C. Hauser, Geneva; C. Eberhard Klein, Würzburg)***

16:00–16:20 C. Foster, Vienna: Introduction
16:20–16:40 A. Sonnenberg, Amsterdam: The role of the $\alpha_6\beta_4$ integrin and BP180 in epidermal cell adhesion
16:40–16:55 G. Zambruno, Rome: Keratinocyte "emergency" integrins during human wound healing
16:55–17:10 C. Eberhard Klein, Würzburg: Adhesion receptors involved in melanoma cell motility and invasion
17:10–17:25 S. Simon, Freiburg: CD44 isoform expression by human Langerhans cells: differential regulation during migration and functional relevance for lymphocyte homing
17:25–17:55 Poster discussions
17:55–18:00 C. Eberhard Klein, Würzburg: Concluding remarks

Tuesday, October 1, 1996

08:15–10:30 ***NON-CONCURRENT MINISYMPOSIUM: Langerhans cells (chairpersons: G. Stingl, Vienna; D. Schmitt, Lyon)***

08:15–08:35 N. Romani, Innsbruck: Life and death of a Langerhans cell
08:35–08:50 D. Strunk, Graz: Langerhans cell ontogeny—a distinctive pathway of dendritic cell development
08:50–09:05 S. Aiba, Okayama: Activation of Langerhans cells
09:05–09:20 G. Zambruno, Rome: Langerhans cells—a target of HIV infection
09:20–09:35 A. Enk, Mainz: Cytokine-induced modulation of Langerhans cell function
09:35–09:50 G. Girolomoni, Rome: The skin—a source of dendritic cell lines
09:50–10:05 J. Peguet, Lyon: Accessory molecules in Langerhans cell antigen presenting function
10:05–10:30 Poster discussions

10:30–11:00 *Coffee*

11:00–13:00 ***Plenary oral communications (Chairpersons: W. Sterry, Berlin, ESDR President, K. Thestrup-Pedersen, Aarhus, President-elect ESDR)***

11:00–11:15 **Stelwagon-Awardee Lecture**
11:15–11:30 W. H. Irwin McClean *et al.* Complete cDNA Sequence, genomic organisation and chromosomal localisation of the human plectin gene
11:30–11:45 Mosaad Megehed *et al.* Cloning, genomic organization and chromosomal mapping of the human and mouse linear IgA disease genes (LAD)
11:45–12:00 K. Scharffetter-Kochanek *et al.* A polygenic mouse model for psoriasiform skin disease
12:00–12:15 Pierre A. de Viragh *et al.* Transient congenital erythroderma in loricrin deficient mice
12:15–12:30 A. Bouloc *et al.* Dendritic cells in intracutaneous DNA immunization
12:30–12:45 F. Koszik *et al.* Loss of tumorigenicity of interleukin 2 producing M-3 melanoma cells is due to infiltrating leukocytes: NK-cells play a major role
12:45–13:00 Gary J. Fisher *et al.* UVB activates stress-activated protein kinases, FOS/JUN and FOS/JUN driven dermis-degrading proteinases in human skin *in vivo*
13:00–13:15 Richard Roden *et al.* *In vitro* generation of infectious papillomavirus and assessment of serological cross-reactivity

13:15 **Poster awards and closure of meeting**

ABSTRACTS

PLENARY ORAL COMMUNICATIONS 1-8
Chairpersons: W. Sterry, Berlin, President ESDR
K. Thestrup-Pedersen, Aarhus, President-elect ESDR

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1

COMPLETE cDNA SEQUENCE, GENOMIC ORGANISATION AND CHROMOSOMAL LOCALISATION OF THE HUMAN PLECTIN GENE.

W. H. Irwin McLean[1], Frances J. D. Smith[1], Elizabeth L. Rugg[1], Leena Pulkkinen[2], Florencia Bullrich[3], Irene M. Leigh[4], Robin A. J. Eady[5], E. Birgitte Lane[1] and Jouni Uitto[2] [1]CRC Cell Structure Research Group, Dundee; [2]Dept Dermatology and Cutaneous Biology and [3]Kimmel Cancer Center, Philadelphia; [4]Experimental Dermatology, and [5]St John's Institute of Dermatology, London.

Hereditary deficiency of the cytoskeletal cross-linking protein plectin is known to occur in epidermolysis bullosa with muscular dystrophy (EB-MD). To allow mutation detection in EB-MD, we cloned the human plectin cDNA and gene. A novel PCR based strategy was used to clone and sequence the 14.8 kb cDNA sequence encoding 4574 amino acids. The calculated molecular weight of the human plectin polypeptide is 518 kDa. One alternatively spliced transcript was also detected. P1 genomic clones were isolated by PCR screening and contained sequences from both ends of the cDNA. Direct sequencing of P1 DNA and long range PCR fragments resulted in determination of the genomic organization. The gene (PLEC1) consists of 33 exons spanning ~26 kb. Two of these exons are unusually large (about 3.3 and 7.3 kb), and consequently, the gene is very compact. The gene was mapped to 8q24 by FISH. The human sequence extends further 5' of the previously reported rat mRNA sequence. This additional sequence encodes a domain with high homology to spectrin and α-actinin, placing plectin in the dystrophin family of proteins. The coiled-coil segments of the central rod domain are highly conserved between rat and human plectin. The carboxy terminal globular repeat domain which is thought to be involved in intermediate filament associations is conserved between plectin, desmoplakin and BPAG1. Plectin functions as an actin and intermediate filament binding protein and its deficiency may explain the combined phenotypes of epidermal fragility and muscular dystrophy in EB-MD.

2

CLONING, GENOMIC ORGANIZATION AND CHROMOSOMAL MAPPING OF THE HUMAN AND MOUSE LINEAR IgA DISEASE GENES (*LAD*).

Mosaad Megahed, Kiyohisa Motoki, John McGrath, Sal LaForgia, and Jouni Uitto, Department of Dermatology and Cutaneous Biology, Jefferson Medical College, Philadelphia, PA; St. John's Institute of Dermatology, St Thomas' Hospital, London, UK.

Linear IgA disease is an acquired autoimmune blistering disorder characterized by circulating IgA autoantibodies directed against the cutaneous basement membrane zone. These antibodies identified in most reported cases a 97-kDa anchoring filament protein. In this study, we cloned the human gene that encodes this autoantigen, designated ladinin, as well as its mouse homologue. The patient's serum, which was used for immunoscreening of a human keratinocyte λgt11 cDNA expression library, bound to anchoring filaments in immuno EM and recognized a 97-kD polypeptide in immunoblotting. Similar results were obtained with antibodies raised in rabbits against a fusion protein corresponding to 315 amino acids of the deduced peptide sequence. Analysis of the amino acid sequence of ladinin revealed that it is a basic secretory protein with eight repeats of a tripeptide SEK motif, an N-terminal basic amino acid stretch, and 4 potential glycosylation sites. The mouse gene demonstrated 69.8% identity at the deduced amino acid level and 75.1% identity at the nucleotide level. Both the human and mouse genes are single copy within the genome, located on chromosome 1, and consist of 10 exons. Northern blot examination showed that the human *LAD* gene is expressed in epidermal keratinocytes, kidney, pancreas, placenta, liver, lung, prostate, small intestine, colon, testis, and ovary, as well as in skeletal and cardiac muscles. Elucidation of the primary structure of the protein serving as 97-kDa autoantigen in linear IgA disease may help in revealing the pathogenesis of linear IgA disease. Also, evolutionary conservation of certain structural features in the mouse gene attests to the importance of this protein as a structural component of the cutaneous basement membrane zone.

3

A POLYGENIC MOUSE MODEL FOR PSORIASIFORM SKIN DISEASE.

K. Scharffetter-Kochanek, D.C. Bullard, J. McArthur, J.G. Chosay, M.E. McBride, C. Montgomery, A.L. Beaudet. Depts. Dermatology, Univ. Cologne, Germany, Mol. & Hum Genetics, Dermatol., Comp. Med., Baylor Coll. of Med. & Howard Hughes Med. Inst. TX, & Dept. Cell Biol. & Inflamm. Res., Upjohn Laboratories, Kalamazoo, MI, USA

A variety of inflammatory skin disorders are known to have a genetic basis, although in many of those diseases the number of genes involved and their pathogenic role remains to be defined. We describe for the first time a polygenic inflammatory skin disease model in CD18 (β_2 integrin) deficient mice with histological and clinical similarities to human psoriasis. Using an insertion vector for gene targeting, CD18 deficient mice of the C57 BL/6 and 129 SV genetic background have been generated displaying up to 16% of the normal CD18 levels and no spontaneous skin phenotype. However, when this mutation was backcrossed onto the PL/J inbred strain, all homozygous mice developed a chronic inflammatory skin disease. The failure to identify bacterial, viral or fungal organisms and the resolution of the dermatitis after subcutaneous administration of dexamethasone suggest the involvement of an autoimmune or other inflammatory process. The level of CD18 expression by itself appears to be not sufficient to precipitate the disease because no skin phenotype was observed in a complete CD18 knock-out mouse. In order to assess the role of other genetic loci, homozygous mutant mice on the susceptible PL/J and resistant C57 BL/6 background were intercrossed. None of the F1 developed dermatitis, but 50% developed dermatitis when F1 mice were backcrossed with CD18 deficient mice on the PL/J strain suggesting that, in addition to the CD18 mutation, a single locus determines the susceptibility to the dermatitis. This model may have general relevance to polygenic human inflammatory diseases, and will help to identify genes, which interact with the β_2 integrins (CD18) in inflammatory processes.

4

TRANSIENT CONGENITAL ERYTHRODERMA IN LORICRIN DEFICIENT MICE.

Pierre A. de Viragh[1,2], Lis Schärer[3], Donnie Bundman[3], Dennis R. Roop[2,3]. [1]Dept. of Dermatology, University Hospital Lausanne/Switzerland, Depts. of [2]Dermatology and [3]Cell Biology, Baylor College of Medicine, Houston/USA.

Loricrin is a major precursor of the cornified cell envelope of terminally differentiated keratinocytes and accounts for greater than 50% of its protein. It is thus thought to play a crucial role in formation of a functional cell envelope and in maintenance of epidermal barrier function. Further, loricrin is presumed to interact with the keratin cytoskeleton of keratinocytes and thus to contribute to the mechanical integrity of the epidermis. To generate mice null for loricrin and to assess its functional importance *in vivo* we deleted by homologous recombination in one allele of embryonic stem cells a 2.5 kb PstI/PstI-fragment at the loricrin locus, encompassing the entire coding region of 1.4 kb. These cells were injected into host blastocysts and participated in the generation of chimeric mice with germline transmission of the mutated allele. Their offspring, heterozygous for the ablated loricrin gene, were bred to generate mice homozygous for the null allele. Absence of loricrin in the homozygous mice was confirmed at the RNA level by Northern blotting, and at the protein level by Western blotting, and by immunohistochemistry. The loricrin knockout mice suffer from congenital erythroderma with a shiny, translucent skin, and are compromised in weight. Their skin is in a state of regenerative hyperproliferation, as is inferred from the expression of keratin 6 and by an 2-3 fold increase of BrDU labbeled cells in the epidermis. Within days the pups heal spontaneously. The skin of adult loricrin deficient mice does not differ from skin of normal litter mates. It appears that loricrin is replaced in knockout animals by the upregulation of other cell envelope precursors. Thus, the pool of cornified envelope precursors constitutes a redundant system to ensure the integrity of the cell envelope even in instances of loss of one of the major precursors.

5

DENDRITIC CELLS IN INTRACUTANEOUS DNA IMMUNIZATION.

A. Bouloc, P. Walker, J.C. Grivel, J. Vogel, S.I.Katz. NCI, NIH, Bethesda MD.

DNA vaccination is accomplished by injecting plasmid DNA encoding proteins into target tissues. Although this method has proven to be effective in animal models, the mechanisms by which this immunization occurs are unknown and the role that antigen presenting cells play has not been explored. Because injection of DNA into skin results in the expression of the encoded proteins, we determined whether intradermal *(ID) injection of DNA could induce sensitivity and, if so, whether dendritic cells* (DC) play a role in inducing sensitization to the protein encoded by the DNA. ID injection of a CMV promoter-driven DNA plasmid encoding hen egg lysozyme (pCMV:HEL) induced expression of immunoreactive HEL protein in the epidermis and dermis. Both *humoral (IgG antibodies-titer 1:2560) and cellular responses* (antigen specific proliferation [stimulation index=3H-TdR in HEL+lymph node cells (LNC) from pCMV:HEL mice/3H-TdR in HEL+LNC from plasmid vector alone=4.3 to 11.2], and induction of CTL) were observed in Balb/c, C3H and C57BL/6 mice. Dendritic cells that migrated out of skin organ culture 2 or 3 days after ID injection were assessed for antigen presenting function in vitro and in vivo. Migratory DC from pCMV:HEL injected skin induced a 2.8 to 6.7-fold increase in 3H-TdR incorporation by CD4+ T cells obtained from HEL protein sensitized animals compared to that *induced by the same number* (50,000) of DC obtained from mice injected with the control plasmid vector. Adoptive transfer of migratory DC from pCMV:HEL injected skin sensitized naive syngenic mice (4.7 to 11.1-fold increase comparing 3H-TdR incorporation in the presence of HEL vs. cytochrome c and priming of MHC class I-restricted HEL-specific CTL). Finally, migratory DC from mice injected with pCMV:HEL contained mRNA encoding HEL (RT-PCR). These results indicate that ID injection of DNA encoding HEL leads to association of nominal HEL antigen with cutaneous DC that can initiate primary and secondary responses, and suggests that DNA-derived antigen may be synthesized within DC.

6

LOSS OF TUMORIGENICITY OF INTERLEUKIN 2 PRODUCING M-3 MELANOMA CELLS IS DUE TO INFILTRATING LEUKOCYTES: NK-CELLS PLAY A MAJOR ROLE

F. Koszik[o], A. Schneeberger[o], W. Schmidt*, S. Strommer[o], R. Kutil[o], G. Stingl[o]. [o]DIAID, Dept. of Dermatology, Univ. of Vienna Med. School, Vienna, Austria; * Research Institute of Molecular Pathology, Vienna, Austria.

Upon transfection with the IL-2-gene, murine M-3 melanoma cells lose their tumorigenicity in euthymic as well as athymic mice. To determine whether IL-2 gene expression directly interferes with the growth properties of M-3 cells in vivo, or alternatively, exerts its effect via the production *of tumor-suppressive/-destructive host factors, we injected IL-2-gene transfected M-3 (M-3-IL-2)* cells and, for control purposes, non-/mock-transfected cells into irradiated (5 Gy) and non-irradiated DBA/2 mice and monitored tumor growth. Whereas all recipients of non-/mock-transfected M-3 cells developed rapidly growing tumors, s.c. inoculated M-3-IL-2 cells were non-tumorigenic in untreated hosts but led to the appearance of slowly growing tumors in irradiated animals. Together with the finding that all types of M-3 cells used in this study exhibit similar growth rates in vitro, this observation implied that M-3-IL-2 cells induce an anti-tumor response in radiosensitive host cells. Indeed, immunohistology revealed a dense leukocytic (MΦ >Gran.>NK) infiltrate (6000 $CD45^+$ cells/mm^2) around and within M-3-IL-2 inocula. In addition, we observed a rapid decline in the size of the *M-3-IL-2 foci over a course of 8 days. To identify the cell* population(s) responsible for this phenomenon, we coinjected (Winn-Transfer) M-3-IL-2 and, for control purposes, M-3 cells together with unstimulated peritoneal lavage cells (PLC) into irradiated, i.e. immunosuppressed host animals. Whereas recepients of M-3-IL-2 cells or of M-3 cells+PLC developed tumors, mice that had received M-3-IL-2 cells + PLC remained tumor-free for 7 weeks. The protective effect of PLC was also seen when this cell population was first depleted of adherent cells, i.e. mononuclear phagocytes. In contrast, in vitro depletion of NK-cells by anti-asialo-GM1 treatment led to significant growth of M-3-IL-2 cells in athymic, nude mice. Our data demonstrate that IL-2 gene-transfected melanoma cells can induce a tumoricidal leukocytic host response and support the notion that *NK-cells play a decisive role in this process. Because tumor cell fragments* may act as critical immunogens when presented in the context of accessory cells, these observations may have important implications for the design of clinically effective cytokine-based cancer vaccines.

7

UVB ACTIVATES STRESS-ACTIVATED PROTEIN KINASES, FOS/JUN AND FOS/JUN DRIVEN DERMIS-DEGRADING PROTEINASES IN HUMAN SKIN *IN VIVO*. Gary J. Fisher, Harvinder S. Talwar, Zeng-Quan Wang, Fiona McPhillips, Sewon Kang, and John J. Voorhees, Department of Dermatology, University of Michigan, Ann Arbor, Michigan.

Exposure of human skin to ultraviolet B irradiation (UVB) results in rapid upregulation of transcription factor AP-1, and induction of AP-1-regulated metalloproteinases (MMP) genes, collagenase, stromelysin-1, and 92kDa gelatinase. MMP-mediated dermal extracellular matrix degradation is likely a critical mediator of sun-induced premature skin aging (photoaging). We have investigated: 1) signal transduction pathways leading to activation of AP-1; 2) expression and UVB regulation of jun and fos family members, which comprise AP-1; and 3) localization of MMP mRNA, protein, and activities induced by UVB in human skin. UVB (2MED) rapidly activated the stress-activated protein kinases (SAPK), JNK, and p38. JNK and p38 activities were elevated 2-fold within 1 hr, and maximally elevated (6.4+1.2 $p<.01$ and 6.4+0.7 $p<.01$, respectively), within 4 hrs. In contrast, no induction of the mitogen-activated protein kinases (ERK-1 and ERK-2), was detected, within 8 hrs following UVB treatment. In untreated human skin, AP-1 was composed predominantly of jun D and c-fos. UVB induced c-jun, jun B, and fra-1 mRNA and proteins. c-Fos and jun D protein levels remained unchanged. Maximal induction of c-jun (3.5 ± 1.0, $p<.01$), jun B (2.6 ± 0.2, $p<.01$), and fra-1 (2.8 ± 0.2, $p<.01$) proteins occurred 4-8 hrs post UVB. Gel shift and supershift analyses reavealed increased c-jun-containing AP-1 complexes 4-8 hrs post UVB. MMPs mRNA, proteins, and activities were maximally induced 16 hrs post UVB. Induction of MMPs by UVB occurred throughout the epidermis and in upper dermal cells, as revealed by *in situ* hybridization and immunohistology. MMP activities were also induced in the epidermis and dermis, as determined by *in situ* zymography. These data are consistent with a model whereby UVB activates the SAPKs, JNK and p38, which act on their substrates c-jun and ATF-2 resulting in increased c-jun gene transcription. Newly synthesized c-jun heterodimerizes with constitutively expressed c-fos, resulting in active AP-1 complexes, which stimulate transcription of MMP genes in human skin *in vivo*. These data define a sequence of UVB-induced molecular events leading to dermal matrix destruction, which would be expected to ultimately result in premature skin aging.

8

IN VITRO GENERATION OF INFECTIOUS PAPILLOMAVIRUS AND ASSESSMENT OF SEROLOGICAL CROSS-REACTIVITY.

Richard Roden‡, Reinhard Kirnbauer*, Doug Lowy‡, John Schiller‡, ‡Laboratory of Cellular Oncology, NCI, Bethesda, MD, USA, Division of Immunology, Allergy and Infectious Diseases (DIAID), Department of Dermatology, Univ. of Vienna, Austria.

Studies of the human papillomaviruses (HPV) have been hampered by the inability to generate sufficient amounts of virions and by the lack of a quantitative in vitro infectivity assay. This deficit has been partially overcome by the expression of papillomavirus L1 and L2 capsid genes in eukaryotic cells demonstrating self-assembly into virus-like particles (VLP). These capsids express conformational neutralizing epitopes and induce type specific protective immunity against cutaneous as well as mucosal papillomavirus (PV) challenge in animal models.

In order to evaluate the potential for cross-protection in a polyvalent HPV-VLP vaccine to prevent high risk genital HPV infection, we developed a system to generate infectious papillomavirus. Cultured cells harboring autonomously replicating bovine PV (BPV) genomes were infected with recombinant Semliki Forest Virus vectors encoding BPV L1 and L2 capsid proteins, leading to the generation of infectious BPV virus. The encapsidation process is sufficiently conserved among papillomaviruses that expression in the same cells of the L1 and L2 genes from HPV16, the type most frequently found in cervical cancer, generated infectious pseudotypes that have the BPV DNA encapsidated by the HPV16 L1 and L2 proteins. These HPV16 pseudotypes induce focal transformation of mouse fibroblasts that are indistinguishable from foci induced by authentic BPV. The infectivity of these HPV16 pseudotypes can be specifically neutralized by antisera generated against HPV16 VLP but not by sera raised against BPV VLP or even VLP of the most closely related HPV types 31 and 33, strongly suggesting that protection in an HPV vaccine will be genotype specific. This assay should facilitate rational design of an HPV vaccine and provide insight into the mechanisms of papillomavirus encapsidation.

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RECOMBINANT ADENO-ASSOCIATED VIRUS (rAAV) VECTORS ALLOW HIGHLY EFFICIENT GENE TRANSFER INTO KERATINOCYTES. M. Braun-Falco, R. Buhmann, A. Doenecke, R. Schilling, E.-L. Winnacker, M. Hallek. Genzentrum, Ludwig-Maximilians-Universität, München, Germany.

The skin has become an attractive target tissue for human somatic gene therapy. However a major limitation for further progress in skin gene therapy is the lack in appropriate gene transfer vectors. Since preliminary results had shown a relatively high transduction rate of rAAV vectors in lung-epithelial tissue, we tested the potential of rAAV vectors for gene transfer into keratinocytes. For this purpose, the gene coding for β-galactosidase (β-gal) under the control of an RSV promoter was constructed in rAAV vectors. Using a recently optimised packaging procedure we achieved a yield of up to 10^6 infectious rAAV particles per ml as determined by dilution experiments in HELA cells. Primary human keratinocytes grown without a feeder-layer were infected with these rAAV/β-gal vector particles used at a M.O.I. of 0.01 to 10. Staining for β-gal activity by cytochemical methods after 72 hours showed a dose-dependent relationship between vector multiplicity and the percentage of cells transduced. At M.O.I. of 10 particles per cell up to 60% of keratinocytes could be reproducibly transduced. Taken together, the results show that rAAV can transduce culture primary human keratinocytes with high efficiency. Since AAV has some important advantages like its low immunogenicity, no pathogenicity for humans, and its potential for long-term expression, further studies are warranted and will show whether rAAV is able to allow long-term transduction of keratinocyte stem cells.

10

UPTAKE AND INTRACELLULAR DISTRIBUTION OF OLIGONUCLEOTIDES IN CULTURED HUMAN KERATINOCYTES. Miriam Wingens, Candida A.E.M. van Hooijdonk, Gijs J. de Jongh, Joost Schalkwijk, and Piet E.J. van Erp, Department of Dermatology, University Hospital Nijmegen, The Netherlands.

Antisense oligonucleotides have the ability to enter living cells and block the expression of specific genes. The efficiency of endogenous uptake varies from cell type to cell type and is essential in the design of therapeutically useful antisense oligonucleotides.

In an approach to develop oligonucleotides as specific inhibitors of skin disease-specific gene expression, we studied oligonucleotide uptake and intracellular distribution in cultured human keratinocytes. For this purpose, we have used a fluorescein-5-isothiocyanate (FITC)-labeled phosphorothioate oligonucleotide, which had a length of 26 bases. Cell-associated fluorescence was followed by flow cytometry and fluorescence microscopy. The oligonucleotide uptake was not uniform among all cells. Approximately 10-20% of cultured keratinocytes took up increased amounts of oligonucleotides compared to the other keratinocytes. Using fluorescence microscopy we showed that in both populations, the oligonucleotides were intracellularly transported and were mainly located in the cytoplasm. Whereas in dead cells the oligonucleotide distribution was frequently intranuclear. We found the uptake was concentration- and time-dependent and the process is hampered by temperature decrease, indicative for an active uptake mechanism. Using flow cytometry the two subpopulations with respect to oligonucleotide uptake were sorted and compared by measuring relative DNA content with propidium iodide. No differences in cell cycle pattern were found between the two subpopulations. So, we conclude that cell entry of oligonucleotides in keratinocytes is not related to cell cycle phase, at least not for the oligonucleotide we have used. Further studies have to be done to determine the basis for heterogeneity in oligonucleotide uptake by human keratinocytes.

These studies are the first step in the understanding of keratinocyte oligonucleotide uptake and are important for the design and interpretation of *in vitro* and *in vivo* experiments with oligonucleotides targeted to specific regions of the RNA and their appropriate controls.

11

MITOCHONDRIAL ACTIVITY AND CADHERIN RECEPTORS AS KERATINOCYTE STEM CELL MARKERS ?

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In vitro, keratinocyte stem cells can be partially purified on the basis of integrin expression and adhesion to the extracellular matrix. In order to develop new keratinocyte stem cell markers, we investigated the mitochondrial activity of cultured keratinocytes as well as the expression of other adhesion molecules.

We used a fluorescent dye, JC-1, as an indicator of mitochondrial activity. We observed that : 1/ Keratinocytes showed an heterogeneity for the mitochondrial activity. 2/ Keratinocytes with high mitochondrial activity had high capacity to form colonies and 3/ These cells localised at the edge of the growing colony. These results suggested that keratinocyte stem cells might be activated during exponential growth.

We analysed the expression of cadherins and catenins, and components of the desmosomes by immunofluorescence and confocal image analysis on human skin sections. We observed that : 1/ Desmocollins, desmogleins, P-cadherin and α-catenin expressions did not vary among the basal cells. 2/ High γ-catenin-expressing cells colocalised with the high integrin-expressing cells and 3/ High E-cadherin and β-catenin-expressing cells showed an inverse localisation with the high integrin-expressing cells. These variations might have reflect the different stages of the keratinocyte lineage.

Taken together with the integrin expression, these results might help to follow the keratinocyte behaviour and to purify the different cell populations.

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CYTOPLASMIC PROCESSING OF HUMAN PROFILAGGRIN BY ACTIVE μ-CALPAIN.

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Differentiation of keratinocytes involves numerous steps including formation of the cornified envelope and the aggregation of keratin filaments by filaggrin monomer molecules, with a molecular mass of 37 kDa in human species which are produced by the processing of profilaggrin. The processing of profillagrin to filaggrin, one of the most useful markers for keratinization, occurs in two distinct stages. The latter enzyme is quite similar to calpain in its characteristics.

In this study, by using an anti-active μ-calpain specific antibody, we demonstrated that active μ-calpain with a molecular mass of 76 kDa appeared 96 h after the addition of Ca^{2+} which was preceded by an increase in μ-calpain mRNA. No changes to either m-calpain or calpastatin were observed under the same conditions. Synchronously to this appearance, the processing of profilaggrin occurred. Furthermore, the Ca^{2+}-induced activation of calpain and the processing of profilaggrin were inhibited by the addition of a synthetic calpastatin inhibitor. These results indicate that the Ca^{2+} influx and the activation of μ-calpain play major roles in second stage profilaggrin processing and keratinocyte differentiation.

13

AN APPROACH TOWARDS THE ISOLATION OF ANTIBODIES SPECIFIC FOR *DIFFERENTIATED CULTURED KERATINOCYTES FROM PHAGE* DISPLAY ANTIBODY LIBRARIES.

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It has become possible to isolate recombinant antibodies from libraries of antibody fragments displayed on filamentous bacteriophage, thereby bypassing immunization and hybridoma technology. The specific aim of this work was to establish a method by which phage displayed antibodies can be selected against proteins that are differentially expressed between different cell populations, since such antibodies can be useful in studying the biological processes that are defining the phenotype of the cells. A competitive selection strategy was developed and tested in two different model systems: 1) Using cytosolic extracts from a melanoma cell line, selections were performed towards the cell extract with and without competition from the same extract, and 2) using commercial proteins of high purity, selections were performed to identify antibodies recognizing a defined difference between two otherwise identical protein mixtures. The resulting phage antibodies were analyzed by ELISA and immunoblotting. 1) The selections against melanoma cell extract performed without competition resulted in 67% positive clones, whereas no positive clones could be identified after competitive selections. 2) Following the competitive selections of the second model system, more than 90% of the clones were reactive towards the protein making up the difference. In contrast, no reactivity was observed towards the other proteins. The results confirm the subtractive nature of the panning system, and suggest that the strategy described may be a fast and easy way to obtain antibodies directed against specific biomarkers. Studies are in progress to identify antibodies that bind specifically to cytoplasmatic proteins of cultured differentiated keratinocytes.

14

BENZYLESTER HYALURONIC ACID MEMBRANES AS SUBSTRATE FOR CULTIVATION AND TRANSPLANTATION OF AUTOLOGOUS KERATINOCYTES FOR THE TREATMENT OF NON-HEALING WOUNDS

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Cultured keratinocyte sheets have been used previously to treat various insufficiently healing wounds. The disadvantages of these grafts are obvious. The culturing procedures are labor intensive, time consuming, and the final tissue layers proved to be extremely fragile and difficult to handle. We present an alternative keratinocyte carrier system for the treatment of non-healing wounds. The patient, a 54 year old female, had been treated for a primary squamous carcinoma. Because of arterial occlusive disease with stenosis of the left superficial femoral artery, a venous femoropopliteal reversed bypass was installed. Despite surgery progressive mummification and necrosis occurred. Keratinocytes were isolated from a skin biopsy and cultivated on a benzylester hyaluronic acid membrane (Laserskin®, Fidia Advanced Biopolymers srl, Abano Terme, Italy; courtesy of MARKA GmbH, Frankfurt, Germany). Three weeks later the Laserskin-keratinocytes were semiconfluent and could be grafted. After eight weeks the wound should almost complete healing. The successful transplantation of semiconfluent keratinocytes on Laserskin demonstrates the advantage of this method in that these keratinocytes stimulate underlying cells leading to granulation and progressive wound healing.

15

CULTURE OF RECONSTRUCTED EPIDERMIS IN SERUM FREE MEDIUM AT 33°C SHOWS A DELAYED EPIDERMAL MATURATION, PROLONGED LIFESPAN AND IMPROVED STRATUM CORNEUM.

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One of the main limitations of reconstructed epidermis until present is that the tissue architecture rapidly deteriorates after 2 weeks in culture as homeostasis is not maintained.

We have cultured reconstructed epidermis in serum free medium at 33°C. Such cultures show a delay in epidermal maturation and a prolonged lifespan for up to 4 weeks: both the number of living cell layers decrease and the stratum corneum thickens at a slower rate as compared to cultures grown in standard serum containing medium at 37°C. Furthermore the rate of proliferation (as shown by Ki67 immunohistochemical staining) remains constant for the first 3 weeks of culture and is comparable to native epidermis, showing only a slight decrease after 4 weeks in culture. In contrast, cultures grown in serum containing medium at 37°C are extremely hyperproliferative up until the 1st week of culture and then proliferation decreases rapidly to a very low level after 3 weeks in culture and is entirely absent in 4 week old cultures.

In addition, cultures grown in serum free medium have a considerably lower triglyceride content than cultures grown in serum containing medium and this low triglyceride content is comparable to native epidermis. The organization of the stratum corneum lipids in serum free cultures, as determined by SAXD, is also markedly improved.

These results indicate that culture of reconstructed epidermis in serum free medium at 33°C, although not yet optimal, is greatly improved when compared to similar cultures grown in serum containing medium at 37°C.

16

NEW COMPUTER ASSISTED METHODS FOR KERATINOCYTE SHAPE AND MOTILITY ANALYSIS: *IN VITRO* TOOLS FOR CANCER RESEARCH.

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To reduce animal experiments in testing metastatic cell behaviour we developed a new *in vitro* system using computer assisted image analysis. Higher motility of epidermal keratinocytes is directly connected to metastasis of squamous cells. To determine the metastatic potential of cell populations cells have to be injected into a sufficient number of animals and the resulting tumours have to be examined. Our system is based on the observation that one cell type exhibits specific reproducable patterns in motile behaviour. These patterns, now detectable by computer-assisted and mathematic approaches, are effected by transformation to metastatic phenotypes. Therefore we compared normal human keratinocytes (nHEK) and papilloma virus-transfected HEK (trHEK) by analyzing phase contrast image sequences. Due to *one-dimensional* analysis along section lines exploiting the clear-cut dark manifestation of ruffles and cell edges, typical extension, velocity, and regularities in ruffle formation are now accessible to quantitative analysis. In a *two-dimensional* approach automatically digitized cell oulines were analyzed to detect spatio-temporal patterns and coordination of lamellar extension and retraction. High periodicity in motility patterns of lamellipodia and ruffles are remarkable features of these structures. Almost all cells showed regularly alternating pulsations of lamellar protrusions, in some cases even extension waves rotating around the cell periphery. The applied methods have been sensitive enough to detect significant higher motility features of trHEK compared to nHEK.

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CONSTITUTIVE AND INDUCIBLE EXPRESSION OF SKALP/ELAFIN PROVIDES ANTI-ELASTASE DEFENSE IN HUMAN EPIDERMIS.

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Skin-derived antileukoproteinase (SKALP), also known as elafin, is a serine proteinase inhibitor first discovered in keratinocytes from hyperproliferative human epidermis. In addition to the proteinase inhibiting domain which is directed against polymorphonuclear leukocyte (PMN) derived enzymes such as elastase and proteinase 3, SKALP contains multiple transglutaminase (TGase) substrate domains which enable crosslinking to extracellular and cell envelope proteins. Here we show that SKALP is constitutively expressed in several epithelia that are continuously subjected to inflammatory stimuli, such as the oral cavity and the vagina where it co-localizes with type 1 TGase. All epithelia from sterile body cavities are negative for SKALP. In general, stratified squamous epithelia are positive, whereas pseudostratified epithelia, simple/glandular epithelia and normal epidermis are negative. SKALP was found in fetal tissues of the oral cavity from 17 weeks gestation onwards where it continued to be expressed up to adult life. Remarkably, in fetal epidermis SKALP was found from week 28 onwards, but was downregulated to undetectable levels in neonatal skin within 3 months, suggesting a role during pregnancy in feto-maternal interactions or in the early maturation phase of the epidermis. Immunoelectron microscopy revealed the presence of SKALP in secretory vesicles including the lamellar granules. In culture models for epidermal keratinocytes we found that expression of the endogenous SKALP gene provided protection against cell detachment caused by purified elastase or activated PMNs. Addition of exogenous recombinant SKALP fully protected the keratinocytes against PMN-dependent detachment whereas superoxide dismutase and catalase were only marginally effective. These findings strongly suggest that the constitutive expression of SKALP in squamous epithelia, and the inducible expression in epidermis participate in the control of epithelial integrity, by inhibiting PMN derived proteinases.

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EFFECT OF CYTOKINES AND GROWTH FACTORS ON UROKINASE, UROKINASE RECEPTOR, AND PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 mRNAs IN KERATINOCYTE CELL LINES.

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Proteolytic enzymes have various functions in cutaneous biology. During reepithelization of skin wounds, migrating keratinocytes utilize the proteolytic urokinase system to induce the pericellular proteolysis necessary for migration. Urokinase type plasminogen activator (uPA), when bound to its specific receptor (uPAR) on keratinocytes, converts surface bound plasminogen proteolytically to plasmin, which in turn cleaves many extracellular matrix components. UPA activity is controlled by specific inhibitors like plasminogen activator inhibitor 1 (PAI-1). In addition to the complex protein interactions, the uPA system is also strongly regulated on the level of protein synthesis. UPA, uPAR, and PAI-1 are not or barely expressed in normal skin, but are strongly induced in migrating keratinocytes in wounds. In a systematic approach, we have analyzed the effects of cytokines and growth factors relevant in wound healing (IFN-γ, TNF-α, IL-1, IL-6, IL-10, IL-12, TGF-β, TGF-α, bFGF, EGF, glucocorticoids) on uPA, uPAR, and PAI-1 mRNAs expression in the permanent A431 and HaCaT keratinocyte cell lines. Total cellular RNA was prepared 4h after stimulation and analyzed by Northern blotting. Confirming previous observations, uPA mRNA was induced by TNF-α and EGF, and uPAR mRNA was induced by TGF-α and prednisolon. We also report, to our knowledge for the first time, the induction of uPAR and uPA mRNA by IL-10 and IL-12 in keratinocyte cell lines. While TGF-β strongly induced PAI-1 mRNA in HaCaT cells, the uPAR mRNA signal was decreased. Thus TGF-β had differential effects on components of the uPA system. These studies provide the basis for the analysis of transcriptional mechanisms by which components of the uPA system are controlled by cytokines and growth factors during wound healing.

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cDNA CLONING AND EXPRESSION OF TRYPSINOGENS IN HUMAN EPIDERMAL KERATINOCYTES

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We have previously showed that tryptic enzymes are present in human stratum corneum and may play an important role in desquamation by catalyzing the degradation of intercellular cohesive structure together with chymotryptic enzymes. In this study, we performed cDNA cloning to identify the tryptic enzymes expressed in human epidermal keratinocytes.

First we designed degenerated PCR primers from consensus sequences of known tryptic enzymes and performed RT-PCR using mRNA from cultured human keratinocytes to obtain selectively amplified cDNA fragments of tryptic enzymes. We obtained one cDNA, of which sequence was almost identical to human pancreatic trypsinogen III, or trypsinogen IV expressed in brain and pancreas. Using this cDNA fragment as a probe, we screened human keratinocyte cDNA library and obtained two cDNA clones. Nucleotide sequence analysis revealed that the one encodes trypsinogen IV and the other, a new type of trypsinogen. The sequence of the new trypsinogen differs from trypsinogen III and IV only in the first exon. We also examined the expression of trypsinogens in cultured human keratinocytes by RT-PCR. Trypsinogen IV and the new trypsinogen were expressed in keratinocytes, while pancreatic trypsinogen I, II and III were not detected. The new trypsinogen was not expressed in brain and pancreas, suggesting a skin specific trypsinogen. In addition, enteropeptidase (enterokinase) that specifically cleaves trypsinogen to yield active trypsin was also expressed in keratinocytes.

These findings demonstrate that the tryptic enzyme in human stratum corneum is trypsin itself, the active form of trypsinogen expressed in epidermal keratinocytes.

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KERATIN mRNA AND PROTEIN EXPRESSION PATTERNS DEMONSTRATE KERATINOCYTE DIVERSITY IN NORMAL EPIDERMIS OF PALMS AND SOLES.

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The epidermis of the palms and soles is functionally and morphologically different from that in other regions and is preferentially involved or spared in certain genetic and acquired skin disorders. In order to gain more information about this region we investigated the pattern of keratin (K) mRNA expression with nonisotopic in situ hybridization using a panel of digoxigenin labelled riboprobes for K 1, 2e, 5, 6, 9, 10, 14, 16 and 17 and correlated the findings with K protein expression determined by immunofluorescence. All analyses were done in duplicate. When arm skin was used for comparison K1, K10 (suprabasal layers), K2e (upper epidermal layers), K5 and K14 (basal layer) showed a similar expression pattern of both mRNA and protein although K2e mRNA staining was most prominent over primary ridges. In epidermal ridges K14 mRNA and protein were also detected in the first suprabasal layer. The expression of K9 mRNA and protein was strong in suprabasal layers of ridged epidermis but absent in non-ridged epidermis. Immunostaining for K6 and K16 was evident in suprabasal cells (discontinuous pattern for K16) and some basal cells (above dermal papillae) in ridged epidermis but negative in non-ridged epidermis. In contrast K6 and K16 mRNA was detected in both ridged and non-ridged epidermis, though staining for both mRNAs was stronger in ridged epidermis. K6 mRNA was expressed in the basal layer and to a smaller extent in suprabasal layers disappearing in upper granular layers, whilst K16 mRNA staining was limited to the basal layer only. K17 immunostaining was seen in some basal keratinocytes in ridged epidermis (above dermal papillae and in the bottom of ridges). K17 mRNA was detected in all basal keratinocytes in ridged epidermis but only in groups of basal keratinocytes in non-ridged epidermis. These findings indicate that in palmo-plantar epidermis the keratinocyte population is heterogeneous and products of certain K genes show qualitative as well as quantitative differences compared with non-ridged epidermis.

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KERATIN EXPRESSION IN DISEASED PALMO-PLANTAR HUMAN SKIN.

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There are several diseases in which the palms and/or soles present with hyperkeratosis, scaling, vesicles and/or pustules. Immunohistochemical techniques with monoclonal antibodies on frozen sections were used to examine the expression of cytokeratins in 13 cases of eczema, 5 cases of keratoderma, 6 cases of psoriasis and 13 cases of chronic palmo-plantar pustulosis.

The pair K5/K14 was seen most strongly in the lower part of the epidermis, sometimes in all, the pair K1/K10 in the suprabasal cornifying epidermis. K6 and K16, indicating a hyperproliferative state, were present in many specimens; they were seen in the lower epidermis, with the basal cells negative for K16 and sometimes also for K6. Expression of K4 and K13 was negative, except for a few patches of K13 in the lower epidermis; expression of K7 and K19 was mostly negative or weak, K18 was negative in the epidermis. The sweat glands and/or ducts reacted with K1, K6, K7, K8, K10, K14, K16, K18 and K19. In most cases Ki-67 reactivity was limited to 1 or 2 basal layers, but in some cases of psoriasis and pustulosis cells higher up were also positive.

In conclusion of this study, it was not possible to distinguish the various diseases examined by their cytokeratin pattern, except for a tendency to express proliferation markers more strongly in psoriasis and pustulosis.

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A FUNCTIONAL RECEPTOR FOR ANGIOTENSIN II ON HUMAN PRIMARY KERATINOCYTES *IN VITRO*

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Apart from its long known cardiovascular and renal actions, angiotensin II (ANG II) has recently been shown to regulate cell proliferation in various tissues. In order to examine whether ANG II influences the proliferation rate of human keratinocytes as well, we first showed the presence of angiotensin receptors on human primary keratiocytes in vitro by radioligand binding studies using [^{125}I]-Sarlle-angiotensin II as the radioligand. Subsequently, we measured ANG II induced [^{3}H]-thymidine incorporation as a marker of mitogenic activity.

Radioligand binding studies revealed a high affinity binding site for ANG II with a Kd of 4.5 nM and a B_{max} of 0.12 nM. [^{125}I]-Sarlle-angiotensin II binding was not competed for by the AT_1-receptor specific ligand Losartan or the AT_2-receptor specific ligand PD 123177, indicating that keratinocytes express a so far unidentified angiotensin receptor subtype. Stimulation of human keratinocytes with ANG II led to a dose dependent (10^{-10} to 10^{-5} M) increase in [^{3}H]-thymidine incorporation, indicating that the ANG II keratinocyte receptor mediates a mitogenic effect. This effects was comparable at 10^{-9} M with [^{3}H]-thymidine incorporation induced by classical growth factors such as EGF (50 ng/ml) and FGF (50 ng/ml).

These results demonstrate for the first time a possible physiological role for ANG II in human skin involving the regulation of keratinocyte proliferation.

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MHC CLASS II$^+$ KERATINOCYTES EXPRESS INVARIANT CHAIN AND HLA-DM MOLECULE GENES.

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CD4$^+$ T lymphocytes recognize peptide antigens in association with MHC class II molecules expressed on the surface of antigen presenting cells. Keratinocytes can synthesize and express MHC class II antigens after induction with interferon-γ (IFN-γ) both *in vivo* and *in vitro*. Assembly of class MHC II molecules with exogenous peptides requires accessory functions provided by the invariant chain (Ii) and the recently described HLA-DM proteins. The constitutive and IFN-γ-inducible expression of MHC class II, HLA-DM and Ii genes is under the control of a trans-acting transcription factor, CIITA. In this study, we have examined the capacity of cultured human keratinocytes and epithelial cell lines to express HLA-DR, HLA-DM, Ii, and CIITA molecule genes in response to IFN-γ treatment. Moreover, we have investigated the capacity of keratinocytes to express SDS-stable HLA-DR dimers on the cell surface, a property that class II molecule acquire when their groove is properly loaded with peptide. Cultured keratinocytes, as well as NCTC epithelial cells, treated *in vitro* with IFN-γ were shown by RT-PCR analysis to express the two spliced forms of Ii (p33 and p41) and HLA-DMB molecules, and the trans-activator CIITA, with transcripts similar to those present in unstimulated Raji B cells. HLA-DMA mRNA was detected also in unstimulated keratinocytes and upregulated upon treatment with IFN-γ. In contrast, the A431 keratinocyte-like cell line expressed constitutively all the molecules analyzed. Finally, Western blotting experiments showed that HLA-DR molecules present on IFN-γ-treated keratinocytes were remarkably resistant to SDS denaturation at ambient temperature. These results suggest that keratinocytes exposed to IFN-γ can generate and export functional class II-peptide complexes on their surface.

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THE NOVEL ANTIPROLIFERATIVE SYNTHETIC COMPOUND GLYCEROGLYCOPHOSPHOLIPID INHIBITS PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN HUMAN KERATINOCYTES

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Recently the synthesis of a novel phospholipid analogue with antiproliferative properties was published (1). In the present study, we investigated the effect of this glyceroglycophosphoplipid (GlcPC) on the biosynthesis of phosphatidylcholine (PC) as a possible mechanism for inhibiting the growth of keratinocytes and of HaCaT cells *in vitro*. Using radiolabeled choline, the PC biosynthesis was determined under stimulated and non-stimulated culture conditions. After 1 -6 h, it was shown that indeed GlcPC inhibited the incorporation of choline into PC in a dose- and time-dependent manner with a IC_{50} of 10 μM. This inhibition was antagonized under stimulation of PC biosynthesis by oleic acid (200 μM). These data indicate that PC biosynthesis is one target of GlcPC, representing a possible mechanism for the antiproliferative effect of this analogue in both cell populations studied. This is in agreement with previous results obtained by our group with other synthetic phospholipids acting on renal epithelial cells and human keratinocytes (2,3). Based on these data we believe that inhibition of PC biosynthesis may be a common mechanism leading to the inhibition of growth of various cell types.

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ENDOTHELIAL CELLS FROM HUMAN BLOOD CAPILLARIES ALSO EXPRESS THE EPIDERMAL FATTY ACID-BINDING PROTEIN. G. Hagens, I. Masouyé, M.S. Pepper[1], P. Madsen [2], J.H. Saurat, J.H. Veerkamp[3], G. Siegenthaler. Department of Dermatology, University Hospital, [1] Department of Morphology, University Medical Center, Geneva, Switzerland ; [2] Institute of Medical Biochemistry, University of Aarhus, Denmark, [3] Department of Biochemistry, University of Nijmegen, Netherlands.

Epidermal fatty acid-binding protein (E-FABP), previously characterized in human keratinocytes, specifically binds fatty acids (FA) and belongs to the superfamily of the 15 kDa retinoid-binding proteins. Its putative function is to regulate intracellular levels of free FA. PAGE-immunoblotting using an antiserum directed against E-FABP revealed that several human tissues express an immunoreactive band of "E-FABP". However, there was no definite proof that the detected band is identical to E-FABP. By reverse transcription polymerase chain reaction (RT-PCR) using messenger RNA isolated from human endothelial cells, Southern blotting and sequencing of the cloned RT-PCR products, we demonstrate that E-FABP is also expressed in these cells. We show by immunohistochemical analysis that E-FABP is expressed in endothelial cells from small vessels located in the dermis and hypodermis. Furthermore, expression of E-FABP is observed in endothelial cells of the microvasculature from heart, striated muscle, small intestine, placenta and renal medulla. In contrast, E-FABP is not detected in endothelial cells from large vessels. The restricted presence of E-FABP in blood capillaries and in keratinocytes, suggests that its ligand might have very specific functions in these two cell types.

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DOWN-REGULATION OF THE 12-HYDROXYEICOSATETRAENOIC ACID RECEPTORS ON KERATINOCYTES UNDER THE HYDROGEN SULPHIDE TREATMENT.

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The main skin eicosanoid - 12-hydroxyeicosatetraenoic acid (12-HETE) is assumed to play an important role in the pathogenesis of inflammatory skin diseases. Since spa water containing hydrogen sulphide exerts therapeutic effects in chronic inflammatory dermatoses such as psoriasis, the influence of the substance on 12(S)-HETE binding to human keratinocytes via specific receptors was studied. Radioligand binding assays were performed to evaluate the 12(S)-HETE binding to the specific receptor on normal human keratinocytes under different treatment conditions. No competitive inhibition of 12(S)-HETE binding was observed in hydrogen sulphide level up to 30 mg/l, however, pretreatment of cells resulted in a decrease of 12(S)-HETE binding. The analysis of saturation curves showed that the inhibition of 12(S)-HETE binding involved both the receptor affinity and the number of receptors per cell. These findings suggest that the effect of hydrogen sulphide in psoriasis or other skin diseases could be partly mediated via an influence on epidermal 12(S)-HETE binding.

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TYROSIN KINASE INHIBITOR *GENISTEIN* ENHANCES CELL-CELL-CONTACT-FORMATION IN E6/E7 TRANSFECTED KERATINOCYTES: A POSSIBLE ROLE OF ADDUCIN IN TYROSIN-KINASE-REGULATION OF CELL JUNCTIONS

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Adducin, a 200 kDa heterodimeric protein, is one of the first proteins present in adhesion junctions after induction of cell-cell-contacts. *In vitro* adducin bundles F-actin and enhances the binding affinity of spectrin to actin. *In vivo* it is associated with the cytoskeleton and actively involved in stabilizing cell-cell contacts. Its localisation in adherence junctions suggests a role in calcium dependent cell-cell contact formation.

We have raised a specific antibody from human red blood cells to demonstrate the presence of adducin in normal keratinocytes (nHEK) and in keratinocytes transfected with genes H6/H7 of human papilloma virus (trHEK).Under high [Ca^{2+}] conditions (1.1mM) adducin is localized at the plasma membrane of nHEK, whereas cultivation in low [Ca^{2+}] medium (0.03mM) leads to a diffuse intracellular distribution. In contrast to nHEK, adducin was not detectable at sites of cell cell-contacts in trHEK even under high [Ca^{2+}] conditions. Incubation with *Genistein*, a tyrosine kinase inhibitor, induced the redistribution of adducin to cell membranes of neighbouring cells. This redistribution was associated with the formation of desmosomes, previously absent in trHEK.

Equilibrated lysates of nHEK and trHEK grown under high and low [Ca^{2+}] conditions, incubated with and without *Genistein* demonstrated equal amounts of adducin by western blotting. Adducin is playing an important role for the regulation of cell-cell contacts and could be a target for tyrosin encoding oncogenes.

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CD40 LIGATION OF HUMAN KERATINOCYTES INHIBITS THEIR PROLIFERATION AND INDUCES THEIR DIFFERENTIATION.

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While CD40-CD40 ligand interactions are known to regulate B cell proliferation and differentiation, much less is known about the role this receptor plays on other cell types, especially those of non-hematopoietic origin. We report here that CD40 is expressed in normal human epidermis in situ, especially on the basal cell layer, and that it is maintained on cultured epidermal basal cells. Immunoprecipitation and SDS-PAGE analysis confirms that CD40 expressed by epidermal basal cells is immunologically related to the B cell CD40. IFN-γ upregulates CD40 expression on cultured keratinocytes, whereas other proinflammatory cytokines, such as IL-1 or TNFα have little effects. Using CD40-ligand transfected L cells (CD40Lc), we demonstrated that CD40 triggering results in an enhanced secretion of both IL-8 and TNFα by cultured epidermal basal cells, suggesting that CD40-CD40L interactions may play a role in amplifying the cutaneous inflammatory reactions. More importantly, we found that keratinocyte proliferation was significantly inhibited when the cells were grown on CD40Lc, as compared to CD32-transfected, or non-transfected, L cells. This inhibitory effect can be reversed substantially by pretreatment of keratinocytes with anti-CD40 mAb. In addition, inhibition of proliferation could be obtained by adding a soluble form of CD40 ligand to the keratinocyte cultures. Interestingly, inhibition of keratinocyte proliferation on CD40Lc correlates with differentiation of the cells, as assessed by morphological analysis and increased profilaggrin content. Collectively, these results demonstrate that CD40 is expressed and functional on human epidermal basal cells and that, on these cells, CD40 ligation may be a signal for limitation of cell growth and induction of differentiation.

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NITRIC OXIDE IS INVOLVED IN THE CANDIDA ALBICANS KILLING ACTIVITY OF KERATINOCYTES

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Human keratinocytes have been reported to exert *Candida albicans* killing activity, but the mechanism implicated in this activity is not known. Since nitric oxide (NO) generated by activated macrophages is a potent antifungal mediator, and keratinocytes have been shown to express the various forms of NO synthase enzymes and to produce NO upon stimulation, we were interested in whether NO may be involved in the *Candia albicans* killing activity of keratinocytes. Freshly separated human keratinocytes and HaCat cell line were used for the studies. The *Candida albicans* killing activity was measured by incubating the keratinocytes with the yeast cells in a ration of 1:2 for 90 min, and the percentage of dead *Candida albicans* cells was determined by staining with methylene blue. NO production of keratinocytes was measured using the modified Griess method. We could show that the *Candida albicans* killing activity of human keratinocytes and HaCat cells could be upregulated by stimulating the cells by UVB, IL-8 or IFNç, and the increased killing activity was accompanied by increased NO production of the keratinocytes. Addition of L-NAME, a competitive inhibitor of NO synthase, completely blocked both the NO production and the *Candida killing* activity of keratinocytes. These results suggest that NO produced in keratinocytes is implicated in the *Candida albicans* killing activity of epidermal cells. The regulation of NO production in keratinocytes might be important in the understanding of the physiological role of the epidermis in the defense to mycotic infections.

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EFFECT OF BURN PATIENT SERUM ON FIBROBLAST AND KERATINOCYTE MORPHOLOGY

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The possibility that burn patient serum could exert a toxic effect on fibroblasts and keratinocytes involved in wound healing has not been investigated previously, despite numerous reports of the toxicity of burn serum on cells from a variety of internal organs. Fibroblasts and keratinocytes were isolated from neonatal foreskins, harvested from 60-80% confluent cultures at passage 2 and 3 respectively, and seeded at $5x10^4$ cells onto collagen coated coverslips. Following 24h culture, the cells were washed and 0.5ml of culture medium plus 10% heat inactivated burn patient serum added. Cells were cultured for 5 days, processed and viewed by scanning electron microscopy. Fibroblasts cultured with 1 or 2 day post-burn serum from patients with full thickness injuries had reduced cell density, reduced cell volume and exaggerated spindle morphology compared with control cells. Keratinocytes cultured with the same sera showed a loss of sheet integrity, and globular-like structures on their surfaces which appeared to be membrane protrusions. Thus, early burn patient serum contains substances which are toxic to fibroblasts and keratinocytes which may affect the subsequent rate of wound healing.

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ANKYRIN AS A MEMBER OF THE ACTIN-BASED CYTOSKELETON IS PRESENT IN HUMAN SPERMATOZOA Konrad Becker, Katrin Schirren, Gerd Haidl, Hans Wilhelm Kaiser, Department of Dermatology, University of Bonn, Germany

Ankyrin is a 206-215 kD globular protein first demonstrated in erythrocytes linking the transmembraneous anion-exchanger to the actin-cytoskeleton. Ankyrin is expressed in several isoforms and known to bind plasma membrane proteins in tissues such as brain, epidermis, intestine, pancreas, prostata and kidney as well as in cells like lymphocytes, Leydig- and Sertoli cells. ANK3 seems to be the main ankyrin in epithelia and exists in isoforms with 215kD and minor splice products of 120 and 105 kD. Ankyrins are supposed to inhibit lateral diffusion of plasma membrane-proteins, further-more they could be involved in signal transduction. Since actin and spectrin have been demonstrated in spermatozoa it is likely that other constituents of the cytoskeleton are present as well.

We raised an antibody against ankyrin from human erythrocytes to look for the presence of ankyrin in human spermatozoa by western blot and immunofluorescence. Human semen was collected, motile spermatozoa were isolated by percoll gradient and smeared on slides or prepared for SDS- page electrophoresis and western blotting.

Antibodies bound to two proteins with 120 and 105 kD in spermatozoa-lysates in western blots. In immunofluorescence ankyrin was present in the mid-piece of spermatozoa where it is co-lokalized with actin. Possible functions of ankyrin are the linkage of integral membrane proteins like ion-channels and bande 3-like proteins, which are described for spermatozoa, to specialized regions in spermatozoan plasma membrane.

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RETINOL METABOLISM DIFFERS IN HaCaT CELLS AND NORMAL KERATINOCYTES AND IS IFLUENCED BY RETINOIDS, CALCIUM AND THE AGE OF CULTURES Hans Törmä, Serge Michel, Ola Rollman, and Anders Vahlquist*, Dep. of Dermatology, Universities of Uppsala and Linköping, Sweden, and CIRD-Galderma, Cedex, France

Spontaneously immortalized keratinocytes (HaCaT cells) differ from normal human keratinocytes by constitutively expressing differentiation markers which are normally suppressed by vitamin A. We therefore analyzed the cellular vitamin A content, retinoid metabolism and expression of retinoid-binding receptors. The retinol content in HaCaT cells was only 1/5 that in normal keratinocytes, but the proportions of 3,4-didehydroretinol and retinyl esters were similar. At least six different retinoid-binding proteins (RARα, RARβ, RARγ, RXRα, CRBPI and CRABPII) were expressed in HaCaT cells but the CRBPI and CRABPII concentrations were abnormally low. Confluent HaCaT cells incubated with [^{3}H]retinol showed a slower accumulation of tracer than normal keratinocytes. After 24 h, ^{3}H activity in saponified cells corresponded to retinol (52 %), 3,4-didehydroretinol (43 %), retinoic acid (1.5 %) and 3,4-didehydroretinoic acid (1.9 %). Incubation with preformed [^{3}H]retinoic acid resulted in a slow formation of of 4-oxo-retinoic acid but no 9-*cis* retinoic acid or 3,4-didehydroretinoic acid. High [Ca^{2+}] in the medium and >12 d of culture enhanced the 3,4-didehydro metabolism of [^{3}H]retinol, whereas preincubation with various synthetic retinoids had the opposite effect. RARβ−agonists inhibited vitamin A metabolism most, but the response varied in the two types of keratinocytes and was independent of RARβ − expression.

In conclusion, the multiple abnormalities in vitamin A processing can help to explain the HaCaT cells´ altered responsiveness to retinoids.

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CYTOCHROME P450 DEPENDENT METABOLISM OF RETINOIC ACID IN CULTURED HUMAN KERATINOCYTES. F.Jugert, I.Notzon and H.F.Merk, Department of Dermatology, Clinic of the RWTH Aachen, Germany

A major pathway of retinoic acid (RA) inactivation is the cytochrome P450 (P450) dependent 4-hydroxylation of these compounds. In this study we were interested whether an oxidative metabolism in human keratinocytes derived from foreskin is present. The cells were cultured in serum-free keratinocyte growth medium [Ca^{++}0.09mM] with bovine pituarity gland extract and epidermal growth factor and they were used in the second or third subculture at subconfluency. 13-cis-RA (10^{-5}M in DMSO [final concentration of DMSO 0.1%]) was added to the medium and incubated over 24 hours in the dark. In the culture medium and in the cells, 13-cis-RA, *all-trans*-RA and their 4-oxo metabolites were determined by automated column switching HPLC-analysis. The presence of keratinocytes led to the formation of 13-cis-4-oxo-RA as well as *all-trans*-4-oxo-RA. The 4-oxo-metabolites are found in the medium whereas 13-cis-RA and *all-trans*-RA are mainly located inside the cells. It has been proposed that P450 isoenzymes influence cell differentiation processes by regulating the steady state levels of ligands that effect growth and differentiation. Our results demonstrate that P450 dependent RA metabolism is present not only in skin homogenates or HACAT-cells but also in normal human keratinocytes under *in vitro* conditions. Other vitamin-derivates e.g. of vitamin D are substrates of this isozyme family as well and it will be of interest whether there is an interaction of the level of P450.

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IN VITRO SCREENING OF NEW RETINOID CANDIDATES FOR THE TREATMENT OF SKIN DISORDERS. Ch.C. Zouboulis, J.O. Sass*, W.-C. Chen, R. Rühl*, H. Seltmann, C. Plum*, H. Nau*, and C.E. Orfanos, Dept. of Dermatology, University Medical Center Benjamin Franklin, and *Inst. of Toxicology and Embryonal Pharmacology, The Free University of Berlin, Berlin, Germany.

The activity of the clinically new retinoid compounds 9-*cis*-retinoic acid (9cRA), 9,13-*dicis*-RA (dcRA), and all-*trans*-retinoyl-β-D-glucuronide (RAG) was examined and compared to all-*trans*-RA (atRA) and vitamin A (ROL) using a standardized ROL-free culture system for HaCaT cells. All retinoids tested showed a narrow concentration range where they developed antiproliferative effects. The first growth inhibitory concentration of atRA and 9cRA were approx. 10x lower than of dcRA, RAG, and ROL. Western analysis showed an enhancement of keratin 19 expression in HaCaT cells by atRA and 9cRA (3x) but not by dcRA, RAG, and ROL (1.5x) at 48-96 h (10^{-6} M). Northern analysis showed on at least 4x higher early induction of CRABP II mRNA levels in HaCaT cells (at 8 h) by atRA, 9cRA, and dcRA compared to RAG and ROL at 10^{-6} M. atRA and 9cRA also induced CRABP II mRNA levels at lower concentrations but dcRA did not. RAG exhibited an increasing induction of CRABP II mRNA with time. HPLC analysis of cellular retinoid metabolism revealed that atRA reached a cellular level of 136x the medium concentration (MC) at 2 h declining to 80-117x after 6 h. 9cRA only reached approx. 8-9x MC at 2-6 h and isomerized in great amounts to atRA. dcRA remained relatively stable in medium not entering the cells. RAG reached a cellular level of 60x MC (6 h) and showed a slow decline in cells and medium with time. Only trace atRA amounts were present after incubation with RAG. In conclusion, 9cRA is a potent retinoid on human keratinocytes comparable to atRA, however its lower intracellular concentration may indicate better tolerability.

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ALL-*TRANS*-RETINOYL-β-D-GLUCURONIDE (RAG) MAY EXHIBIT RETINOID ACTIVITY BY CONTINUOUS RELEASE OF LOW AMOUNTS OF ALL-*TRANS*-RETINOIC ACID (RA). Ch.C. Zouboulis, H. Seltmann, J.O. Sass*, R. Rühl*, C. Plum*, H. Nau*, and C.E. Orfanos, Dept. of Dermatology, UMC Benjamin Franklin, and *Inst. of Toxicology and Embryonal Pharmacology, FU Berlin, Germany.

The activity of RAG was investigated using a retinol (ROL)-free system of HaCaT cell cultures. HPLC analysis revealed quick ROL esterification and decreased ester utilization under the combined RAG/ROL vs. ROL treatment. RAG reached a cellular level of 42x its initial medium concentration (6 h) and showed a slow decline in the cells and in the medium. Only little RA was present after incubation with RAG (27 nM, 96 h). Intracellular RAG amounts slightly increased under the combined RAG/ROL treatment compared to RAG alone (4672 vs. 4171 nM, 6 h), while RA decreased under the combined treatment (12 nM, 96 h). Continuous presence of RAG in medium was required to obtain max. inhibition of HaCaT cell proliferation (-62%, 120 h). While ROL was practically inactive, simultaneous RAG/ROL treatment decreased RAG effectivity on cell proliferation by 12%. Western analysis showed a weak enhancement of keratin 19 expression in HaCaT cells by RAG and ROL (1.5x at 10^{-6} M, 96 h) which was neutralized by combined RAG/ROL. Northern analysis showed a higher induction of CRABP II mRNA levels in HaCaT cells by RAG (12.2x at 10^{-6} M) than by ROL (5.4x at 10^{-6} M) at 8 h which was less than additive under combined RA/ROL. Our results indicate, that RAG represents a precursor for RA which probably exhibit its activity by continuous release of low RA amounts. When simultaneously applied, ROL may antagonize RAG activity by decreasing RA release.

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EFFECTS OF VITAMIN D_3 ON KERATINOCYTE PROLIFERATION AND DIFFERENTIATION IN VITRO: MODULATION BY LIGANDS FOR RETINOID AND TRIIODTHYRONINE RECEPTORS.
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Keratinocytes express receptors for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), all-trans retinoic acid (at-RA), 9-cis retinoic acid (9-cis-RA) and triiodthyronine (T_3). The vitamin D receptor (VDR) can bind to response elements in promotor regions by forming homodimers or heterodimers with the retinoic acid receptor (RAR), the retinoic X receptor (RXR) or the triiodthyronine receptor (T_3R). In this study we investigated whether the capacity of $1,25(OH)_2D_3$ (10^{-10}-10^{-7} M) to inhibit proliferation and stimulate differentiation in human keratinocyte cultures are modified by the RAR ligand at-RA, the RXR ligands 9-cis-RA and CD2809, and the T_3R ligand T_3 (10^{-10}-10^{-6} M). Second passage keratinocytes were grown to approximately 30% confluency before incubation for 4 days with ligands in keratinocyte growth medium supplemented with 3% charcoal-stripped FCS and 0.3 mM Ca^{2+}. Proliferation was measured by spectroscopy adding a dimethylthiazolyl-diphenyl-tetrazoliumbromide (MTT), and differentiation by a cell-ELISA for transglutaminase type 1. Experiments were done with cultures from 5 donors in quatriplicates. At-RA, 9-cis-RA and CD2809 alone dose-dependently increased proliferation and decreased differentiation. In combination with $1,25(OH)_2D_3$, the retinoids partially counteracted the anti-proliferative effect of $1,25(OH)_2D_3$. The differentiation was dose-dependently inhibited by at-RA, 9-cis-RA and CD2809 alone. In combination with $1,25(OH)_2D_3$, the retinoids reversed the pro-differentiating effect of $1,25(OH)_2D_3$ resulting in a net inhibition of differentiation. T_3 had, neither alone nor in combination with $1,25(OH)_2D_3$, any effect on proliferation or differentiation. In conclusion, the retinoids at-RA, 9-cis-RA and CD2809 have similar effects on keratinocyte proliferation and differentiation. The effects of $1,25(OH)_2D_3$ on proliferation and differentiation was reversed by these retinoids. Therefore, ligand-dependent heterodimer formation between VDR and retinoid receptors may not be important for the effects of $1,25(OH)_2D_3$ on keratinocyte proliferation and differentiation in vitro.

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INTERACTION OF RETINOIC ACID AND 1,25-DIHYDROXYVITAMIN D_3 ON THE PROLIFERATION OF NORMAL HUMAN EPIDERMAL KERATINOCYTES.

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Retinoic acid (RA) and 1,25-dihydroxyvitamin D_3 (VD) both act via nuclear receptors of the steroid hormone receptor superfamily and have potent, well described effects on keratinocyte proliferation. It was our aim to study the nature and underlying molecular mechanisms of their largely unknown, combined action in these cells. By tritiated thymidine incorporation assay on preconfluent keratinocyte cultures, we showed that the concentration-dependent inhibition of DNA synthesis by VD was attenuated by all-trans retinoic acid (atRA) (10 to 500 nM) or 9-cis retinoic acid (9cRA) (10 to 1000 nM). The counteracting effect of RA was both concentration (maximal for RA 100nM) and time dependent (maximal with pre-incubation of RA) and was strongest with 9cRA. We confirmed these results by cell counting, fluorimetric DNA measurement and cell cycle analysis by flow cytometry which demonstrated that the G1 arrest induced by VD treatment, was significantly reduced by RA co-treatment. Northern blot analysis indicated that VD 100 nM treatment for 24h lead to a three fold increase of the mRNA level for transforming growth factor β_1 (TGF β_1), an important autocrine negative growth regulator of keratinocytes. This induction was almost totally abolished by RA. The level of mRNA of p21 (a cyclin-dependent kinase inhibitor and downstream target of TGF β_1) was also reduced in RA plus VD as compared to VD treated cells. RA did not induce significant changes in the vitamin D receptor mRNA or in the down regulation of c-myc mRNA by VD. These results indicate that RA is able to decrease the VD effect on keratinocyte proliferation and that TGF β_1 and its downstream regulators should be considered as possible mediators in this interaction.

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MELANOMA IN EAST-FLANDERS: BELGIUM. A PROJECT TO INCREASE THE REGISTRATION OF MELANOMA LESIONS.

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- The age-adjusted incidence rate of melanoma in Belgium rose from 7.9 in 1984 to 11.13 in 1992. The age-adjusted mortality rate increased from 2.49 in 1984 to 4.41 in 1990. The increase in incidence in this period corresponds to an increase of 40.7 %. The increase in mortality corresponds to an increase of 77 %. We assume a serious underregistration of melanoma cases. We also assume an unknown number of skin lesions treated without any histological diagnosis.
- In order to increase the registration of melanoma lesions, and to raise the attention of physicians towards melanoma detection, a programme of melanoma awareness and correct diagnosis is launched in the province of East-Flanders (1,000,000 inhabitants). In the mean time, a quality control programme of histopathological diagnosis (double interpretation of specimens) and clinical diagnosis will be integrated.
- By the year 1998 we hope to have succeeded in our planning, so that a health education campaign can be started in 1999. Overdiagnosis and over-treatment due to an increased awareness will avoided by an increase of diagnostic ability among the physicians and the integration of a referral structure.

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TRENDS IN MORTALITY RATES FROM MALIGNANT MELANOMA IN BELGIUM 1954 - 1990.

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To monitor mortality rates from malignant melanoma in Belgium, the data from all patients who died of malignant melanoma (n = 3,816) were analysed over the period 1954 through 1990.

Age standardised mortality rates increased from 0.64 to 4.89 in men and from 0.88 to 4.41 in women. Multivariate analysis showed that the risk of dying from melanoma (age-adjusted) increased by 17% every 5-year period (RR = 1.17, 95% CI 1.07-1.28). In the age-period model in men, the relative risk of dying from melanoma increased up to 4.6 compared to the earliest time-period. In women, the relative risk increased up to 4. The highest increases are observed among the age groups 40 to 49, 50 to 59 and 60 to 69 years.

The overal achievements in control of malignant melanoma are best evaluated by changes in the mortality rates. The planning of interventional strategies should be guided by estimates of the mortality rates.

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IL-7-GENE-TRANSFER INTO AUTOLOGOUS MELANOMA CELLS FOR VACCINATION - A PILOT STUDY IN PATIENTS WITH ADVANCED MELANOMA.

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IL-7 is able to augment the growth and cytotoxicity of T cells and to generate LAK cells independently of IL-2. The administration of IL-7-gene-transfected tumor cells in mice has been shown to achieve tumor suppression and tumor eradiction. In a first clinical pilot study, patients with advanced mefanoma were vaccinated with IL-7-gene-transfected, autologous melanoma cells. Melanoma cells were transfected mechanically by a gene-gun approach *ex vivo*. Patients were vaccinated s.c. with irradiated cells into all four limbs close to the inguinal and axillary lymph nodes once in week 1,2,3, and 6. Ten patients were treated according the protocol. Clinically, no side effects except of slight flue-like symptoms in two patients until two days after vaccination were noticed. The immunological monitoring of 8 assessable patients revealed a number of changes upon immunization, however, no objective clinical response was observed. In skin tests, no specific DTH-reaction against autologous melanoma cells was found and response to recall antigens (Multitest Merieux) showed a tendency to decrease according to the progress of the disease. All patients showed a decrease in CD3+ cells in the peripheral blood after the first four weeks as measured by flow cytometry. Interestingly, the majority of the patients demonstrated an increase of the spontaneous cytotoxicity (3/8) and of the LAK-activity (6/8) against autologous and allogeneic melanoma cells measured by an MTT-colorimetric assay after the first four weeks of treatment. Peripheral blood of 5 patients were evaluable for a limiting dilution frequency analysis method before and after vaccination. The number of cytotoxic clones and/or the number of proliferative T-cell clones increased in all cases. In conclusion, these data demonstrate the feasibility of a vaccination therapy with IL-7-gene-transfected autologous melanoma cells in advanced melanoma patients. These results suggest that vaccination with gene-modified tumor cells might have a beneficial effect on the immune response of melanoma patients even in their advanced stage.

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TREATMENT OF PATIENTS WITH MALIGNANT MELANOMA WITH A HYBRID CELL VACCINE.

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Treatment of metastatic melanoma remains one of the major challenges in dermatological oncology. Tumor cells may escape the immune surveillance because they do not express signals that are essential for activation of the host immune system such as MHC molecules or co-stimulatory molecules. Activated B cells are among the most effective antigen presenting cells. In addition to expressing high levels of MHC class II antigens they also express high levels of accessory and co-stimulatory molecules. We therefore hypothesized that by fusing tumor cells with activated B cells we may be able to create a hybrid cell that both produced tumor specific antigens and had all the machinery required for antigen presentation and T cell activation. We have prepared single cell suspensions of melanoma metastasis from several patients and fused them with activated allogeneic B cells by electrofusion. Fusion efficiencies were in the range of 30-40% as judged by membrane labelling with fluorescent dyes and subsequent two-colour FACS-analysis. The hybrid cells expressed high levels of MHC class I and II, ICAM-1, B7-1 and B7-2. For evaluating the in vivo efficacy of this vaccine, we have subcutaneously injected several melanoma patients with an individually prepared and irradiated hybrid cell vaccine. Only minor side effects such as low grade fever werde noted. The observed tumor regressions and increased DTH reactions indicate that hybrid tumor cells may be a useful strategy for immunotherapy of cancers such as malignant melanoma.

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REGRESSION OF MELANOMA AND NON-MELANOMA SKIN CANCER IN MICE AFTER TREATMENT WITH THE SUPERANTIGEN STAPHYLOCOCCAL ENTEROTOXIN B.

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Studies from our laboratory have shown that: I) MHC class II negative epidermal tumor cell lines generate a vigorous in vitro T-cell proliferative response when used as antigen presenting cells for the bacterial superantigen Staphylococcal enterotoxin B (SEB) (Häffner et al., PNAS 1996), and 2) T-cells exhibit profound cytotoxicity against these same epidermal tumors when incubated with SEB. These results indicate that, if the in vitro results could be extended to the in vivo situation, SEB might be a useful therapeutic agent for cutaneous tumors. To address this issue, tumor implantation studies were performed using the murine PAM 212 cutaneous squamous cell carcinoma cell line and the murine B16Fl melanoma cell line. In parallel experiments, animals were treated with SEB administered either intratumorally, subcutaneously or intravenously. Intratumoral injection of SEB into PAM 212 tumors in syngeneic BALB/c mice resulted in 60-75% regression of tumors. Similarly, there was a 60% reduction in the growth rate of SEB-treated B16Fl melanomas in syngeneic C57Black mice. The cytotoxic effect was localized to the tumor with little or no evidence of tissue damage to the surrounding skin. When SEB was injected into PAM 212 tumors that had been implanted into athymic nude mice, tumor regression was not observed, indicating that the cytotoxic effect of SEB on tumor cells was dependent on T cells. As to the route of administration, the intravenous route proved to be as effective as intratumoral injection, whereas subcutaneous administration was less efficient. These experiments demonstrate that SEB is highly effective at inducing an anti-tumor T-cell response in mice in vivo and that superantigens may be valuable agents for the immunotherapy of cutaneous tumors.

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ANALYSIS OF DRUG RESISTANCE OF MELANOMA CELLS: PHARMACOLOGICAL CHARACTERIZATION, CROSS-RESISTANCE AND GENETIC REGULATION.

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To develop new strategies for the treatment of malignant melanoma we studied the mechanism of chemoresistance of melanoma cells *in vitro*. Drug resistance to cisplatin (0.01-1 μg/ml), vindesine (0.05-5 ng/ml), etoposide (0.01-1 μg/ml), and fotemustine (1-100 μg/ml) was established in the melanoma cell line MeWo by continuous exposure to cytostatic drugs with different cellular targets. Analysis of drug-resistant sublines regarding their pharmacological characteristics and cross-resistance pattern revealed an up to 26-fold relative resistance against the alkylating agent fotemustine and an up to 36-fold relative resistance against topoisomerase-II-inhibiting etoposide. In comparison to the parental cell line cisplatin and vindesine selection resulted in a 6- and 10.2-fold increased resistance, respectively. In addition, partial cross-resistance was observed in sublines selected independently. Chemoresistance of MeWo cells was not paralleled by a typical multidrug-resistance due to an increased expression of p-glycoprotein, MRP or LRP. However, in comparison to the parental cell line all resistant sublines showed increased levels of *c-fos* and *c-jun* mRNA encoding components of the transcriptional activator AP-1. Expression of the *nur77* homologue *NAK1* was increased in cells resistant to cisplatin and etoposide. Neither expression of *p53* nor the amount of *bcl-2* mRNA was altered in any of the resistant sublines. The results indicate that modulation of signal pathways regulating death and survival factors may contribute to chemoresistance of melanoma cells.

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HÆMATOGENOUS DISSEMINATION OF MELANOCYTES DURING LYMPH NODES RESECTION. **B. DRENO*, M. DENIS**, M.H. TESSIER*, P. LUSTENBERGER****

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Evolution of melanoma is closely correlated with lymph node involvement. Among the patients presenting lymph node metastases, 75% will relapse, and the mean time before distant metastases are detected following node resection is ≈11 months. Thus, detection of circulating melanocytes in peripheral blood might help to select the patients who could benefit from adjuvant therapy after surgery. Therefore, we have looked for melanocytes in blood of melanoma patients with local or regional lymph node metastases (stage IIIB according to MD Anderson). The detection relies on expression of tyrosinase, a key enzyme involved in melanogenesis specifically expressed by melanocytes. Blood samples (10-20 mL) were collected in heparinized tubes. Then extraction of total RNA from these samples and nested RT-PCR were performed. Patients were analysed before and after they underwent lymph node resection.

None of the 16 patients tested 3 days before surgery had detectable circulating melanocytes in their blood. By contrast, 7 of them found to have circulating melanocytes 2-4 weeks after node dissection. No control was positive. One month later, all patients were again negative. Several months after the first resection, one patient developed a regional relapse. Again, lymh nodes were removed. Circulating melanocytes were detected in blood 2 months after this second surgery, and then disappeared. These data were compared with histological characteristics of lymph node involvement. So far no evidence of correlation between released melanoma cells and capsular-breaking or the number of involved lymph node were noted.

Therefore, the cells we detected following lymph node resection can only be released from melanoma metastases. Whether these cells have the potential to develop distant metastases or not is an opened question. A longer follow-up of these patients is required to establish the prognostic significance of this dissemination.

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Attachment of Melanoma Cells to Extracellular Matrix is Inhibited by MIA

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We have recently purified and cloned a small protein secreted into the extracellular compartment from melanoma cells (Blesch et al., 1994). Due to ist antiproliferative activity on melanoma cell lines *in vitro* this protein was designated MIA, melanoma inhibiting activity (Bogdahn et al., 1991). We now report that MIA significantly inhibits invasion of melanoma cells through a reconstituted basement membrane (Matrigel) in Boyden chamber assays *in vitro*. Further analyses revealed that this effect is mediated by interference with attachment of the melanoms cells to laminin and fibronectin. Attachment to collagen type I,II and IV, vitronectin and heparansulfate-proteoglycane was not inhibited. Comparing the MIA effect to the effect of disintegrins, MIA and RGD peptides were added simultaneously to the medium. We observed an antagonistic effect on inhibition of attachment and invasion. The same results were observed using fibronectin or laminin and MIA. These results indicate that MIA functions by a new mechanism. It interferes with the attachment of melanoma cells to a subset of matrix proteins by binding to these proteins and not by binding to the integrins like reported for disintegrins. Consistently, we were able to demonstrate direct molecular interaction of MIA with laminin and fibronectin using a specific MIA immunoassay. Further analysis indicated a direct interaction with distinct region in fibronectin. Our studies point to an important role of MIA in regulating adhesion of melanoma cells to components of the extracellular matrix.

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PERIPHERAL BLOOD mRNA DETECTION OF MART- AND TYROSINASE IN MELANOMA PATIENTS. Werner Löntz, Annett Milling, Wolf Dietrich and Helmut Heise, Department of Dermatology, University of Rostock, Germany.

Recent reports on the RT-PCR based detection of Tyrosinase mRNA in the peripheral blood of melanoma patients point to the great potential of this new diagnostic modality as a staging and surveillance tool in malignant melanoma.

Unfortunately, the wide acceptance of this sensitive technique is still hindered by the frequent occurence of false positive results. In addition, the determination of a single marker may not be sufficient to account for the known genetic heterogeneity of melanoma cells. One approach to verify results by possibly increasing the sensitivity of detection is the application of a multi marker assay. In a first step, we selected the melanoma specific antigen MART-1 as a candidate marker for circulating melanoma cells. A nested PCR approach was used to screen the peripheral blood of 105 melanoma patients for mRNA transcripts of MART-1 and tyrosinase. Prior dilution experiments indicated a sensitivity of about one melanoma cell in 10 ml venous blood for both markers, whereas blood of normal donors was consistently tested negative. Of the melanoma patients, 15 patients had positive results for both markers, 6 were tested positive only for tyrosinase and in 4 patients MART-1 was the single positive marker. The combined results correlated well with disease stage and progression. Stage I patients were positive in 15% (7/45), stage II patients in 23% (9/40). Two of these 85 patients, one who tested positive for both markers and one patient positive for MART-1, developed clinical metastasis during a follow-up period of 6 months. In stage III 39% (7/18) of the patients had positive results. The majority of negatively tested patients of stage III was classified to be either in stable disease or upon chemotherapy during the testing period. Two patients with distant metastasis were found to be repeatetly positive for both markers.

Our analysis confirms the applicability of the RT-PCR assay to monitor disease status in malignant melanoma. The use of MART-1 as an additional specific marker allows in many patients a verification of results, which appears to be necessary before therapeutic decisions can be based on RT-PCR results. In addition, some evidence is provided, that a two- marker system can increase detection sensitivity.

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TYROSINASE-POLYMERASE CHAIN REACTION REEVALUATED. K. Rass, S. Seiter, M. Thome, W. Tilgen. Department of Dermatology, University of Heidelberg, Germany.

Reverse transcription-polymerase chain reaction (RT-PCR) of tyrosinase mRNA facilitates the specific detection of melanoma cells in peripheral blood from patients with metastatic melanoma. Since the first description of this method, strong efforts have been made to determine its clinical value. As previous reports demonstrated contradictory results with sensitivities ranging from 20 to 100% in patients with distant metastases, our approach was to reevaluate this assay. To validate sensitivity 7,5ml blood samples from healthy donors, prepared with defined amounts of SK-Mel 28 cells, were tested. Different techniques of blood preparation, erythrocyte lysis vs. whole blood preparation were compared. Integrity and purity of RNA extracts were checked by agarose gel and spectral photometry, quality of cDNA synthesis by amplifying a housekeeping gene. As nested PCR technique implies a high risk of false positive results, we paid much attention on avoiding contamination and analysed the products very critically. We evaluated blood samples from 30 patients with stage IV melanoma. Titration experiments showed, that it is possible to reproducibly detect 1-5 melanoma cells/ml blood in 64%, 10-50 cells/ml in 82% and more than 100 cells/ml in 100% of the probes by means of lysis-blood technique. Whole blood preparation showed lower sensitivity (19%, 64% and 100% respectively). Using lysis-blood technique only seven patients were found to be positive for tyrosinase mRNA in peripheral blood (23%). Thus, our data support the recent doubts, that RT-PCR of tyrosinase mRNA maybe a highly sensitive tool for the identification of melanoma cells in blood of patients. In our hands this method failed as a prognostic marker in stage IV patients and as a detector of haematogenous spread in earlier stages of melanoma as well.

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CRITICAL EVALUATION OF TYROSINASE AS AN EARLY MELANOMA PROGRESSION MARKER

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Malignant melanoma is known as a tumor with the tendency to metastasize despite radical surgery, suggesting occult melanoma cell dissemination already in an early stage of disease. Recently the reverse transcription-polymerase chain reaction (RT-PCR) of tyrosinase mRNA was reported for detection of circulating tumor cells in the peripheral blood of melanoma patients. Our aim was to establish, optimize, standardize and critically evaluate the diagnostic value of this approach.

The protocols for blood collection, RNA isolation and tyrosinase RT-PCR were validated using different melanoma cell lines (WM 1341, Sk-Mel-23, -25 and -28). Specificity of PCR products was confirmed by restriction enzyme analysis. Subsequently, 112 blood samples from 96 individuals (16 healthy persons, 5 basal cell carcinoma patients, 71 cutaneous, 2 uveal and 2 mucosal melanoma patients) were investigated in a blinded study.

The sensitivity of tyrosinase RT-PCR differed between the cell lines tested. We reproducibly detected single melanoma cells (Sk-Mel-23 and -28) spiked in 10 ml whole blood samples from healthy volunteers. All samples obtained from control persons and patients with non-melanoma skin tumors were negative for tyrosinase mRNA. 1/43 patients with primary melanoma (2,3%), 0/15 patients with regional metastasis (0%) and 3/13 patients with advanced disease (23,1%) were found PCR positive.

These results indicate that tyrosinase RT-PCR fails as a sensitive marker for circulating tumor cells in early stages of malignant melanoma.

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IDENTIFICATION OF MELANOMA CELLS IN „SENTINEL LYMPH NODE" VIA RT/PCR TO DETECT TYROSINASE mRNA IS NOT A MARKER FOR METASTASES OF THE ENTIRE DRAINING SITE.

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„Sentinel node" is the first lymph node of the draining site of a primary tumor. In several types of malignant tumors sentinel node metastases indicates lymph node metastases of the entire draining site. In order to determine whether this approach is applicable to assist staging of malignant melanoma, we investigated lymph nodes of 12 patients by histology and RT/PCR to detect Tyrosinase mRNA, a marker for micrometastases.

Before surgery lymphatic out flow scintigraphy was performed to evaluate the pathways of lymphatic drainage from the site of primary melanoma. Intraoperative patent blue V was injected intracutaneously around the melanoma to visualize the afferent lymphatic ducts and the sentinel node. By combining these methods, the sentinel lymph node could easily be identified in all 12 patients. 6/12 patients had primary melanomas with tumor thickness > 1,0 mm and were considered candidates for elective lymph node dissection. 6/12 patients had melanomas with tumor thickness 0,6-1,0 mm , but Clark level III. In these cases only the marked sentinel node and lymph nodes in its immediate surrounding were removed.

In 11/12 patients histology revealed no metastasis of sentinel node and all the other obtained lymph nodes. 1/12 patient showed metastasis of sentinel node and a positive Tyrosinase PCR. In 3/12 patients Tyrosinase PCR of sentinel node was negativ whereas surrounding lymph nodes gave positive results.

These preliminary results indicate that in melanoma patients the indication for elective lymph node dissection cannot be based on the sentinel node.

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RT-PCR FOR TYROSINASE mRNA IN PERIPHERAL BLOOD DOES NOT SUFFICIENTLY EXCLUDE HAEMATOGENOUS SPREADING OF MALIGNANT MELANOMA

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Previous studies on the detection of circulating melanoma cells in peripheral blood by RT-PCR for tyrosinase gene showed a high variance of positive results ranging between 0% and 100% in stage IV malignant melanoma. Lacking standardized and comparable experimental conditions we developed a strict pattern of analysis consisting of at least 4 double polymerase-chain-reactions (PCR) (using outer primer HTYR-1/-2 and nested primer HTYR-3/-4) following cDNA-synthesis by reverse transcription (RT) of 2 different RNA-extractions from the same blood sample. In all cases of disaccording results we performed further cDNA-synthesis or a third RNA-extraction. Final assessment was considered positive when at least 4 PCR-analyses of 2 RNA-samples gave positive results. Evaluation of 101 patients with malignant melanoma showed a stage-related amount of detectable tyrosinase mRNA in peripheral blood: In 2.5% of all patients with primary cutaneous melanoma (n=40), in 11.4% with regionally spreading melanoma (n=35) and in 40% with distant melanoma metastasis (n=26) circulating melanoma cells were found; whereas, all 20 normal controls studied were negative. The sensitivity of the technique corresponded to 1-5 human melanoma cells SKMel-28 in 5 ml normal blood. Based on a strictly defined pattern of analysis our data demonstrate that RT-PCR for tyrosinase transcripts in peripheral blood of melanoma patients may confirm, but does not sufficiently exclude haematogenous spreading. Therefore, despite the highly sensitive and specific technique and the higher frequence of positive results in advanced clinical stages, its value for early detection of oligocellular micrometastases still remains unsatisfactory.

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LACK OF EXPRESSION OF MELANOMA-ASSOCIATED ANTIGEN FOLLOWING TRANSFECTION IN A HUMAN MELANOMA CELL LINE. Valeria Boccaletti[1], YangMing Yang[2], Zeev Ronai[2], Zhighang Wang[3], Soldano Ferrone[3], Giuseppe De Panfilis[1]. (1) Dept. of Dermatology, Brescia University Hospital, Brescia, Italy ; (2) American Health Foundation, Valhalla, NY and (3) Dept. of Microbiology and Immunology, NYMC, NY, USA.

In melanoma the malignant transformation of cells is associated with the appearance of tumor-associated antigens (TAA). One of them is the high-molecular weight melanoma-associated antigen (HMW-MAA), a chondroitin sulphate protcoglycan expressed on the cell surface. This TAA is classified as an early differentiation marker of the melanocytic lineage, and it is associated to cell adhesion, spreading on substrata, anchorage-independent growth of melanoma cells and cell-cell interactions. Our group tried to inhibit the surface expression of HMW-MAA by transfecting the human melanoma cell line MeWo with the transcriptional factor ATF-2. To assess if the transfection-induced HMW-MAA inhibition, a cell surface study was conducted by means of cell-ELISA and direct binding assays. Results showed a marked down-regulation of HMW-MAA on the transfected lines, whereas MHC-I and II, ICAM-1, VnR and GD3 were still normally expressed, as well as other TAA like 100K- and 115K-MAA. SDS-PAGE carried out with the precleared cell lysate of the transfected cell lines didn't show the characteristic profile of melanoma cells for HMW-MAA. To assess if this was due to a lack of protein synthesis or to an immediate shedding of the protein once it reached the cell surface, a double determinant immunoassay was carried out using as antigen source the cell lysate or the spent medium of the cell culture. This experiment showed an impairment at the post-transcriptional level in the transfected cell lines, which contained similar amounts of antigen, if compaired to the controls. Thus, transfection was able to induce a lack of expression of a functionally important TAA in a human melanoma cell line.

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THE NEW TECHNIQUE FOR PRODUCTION OF TUMOR INFILTRATING LYMPHOCYTES (TIL) : APPLICATION TO MELANOMA-INVOLVED LYMPH NODES- **MC PANDOLFINO*, AUDRAIN M**, ROBILLARD N**, JOTEREAU F***, DRENO B*** Department of cellular and genic therapy ** Laboratory of Immunology *** INSERM 211 Nantes - France.

One of the problem for production of TIL is the risk of viral contamination by using human serum which is necessay for in vitro expansion. To avoid this problem, we used a synthetic medium instead of human serum : X - vivo 15 (Biowhittaker). Thus, with this new method of culture, recently we obtained 10/10 expansion of TIL from melanoma involved lymph nodes (mean of 2.09 10^{10} cells x 2 injections for each patient). The phenotype of TIL was mixed CD4 and CD8 in a variable percentage for each patient. TIL expressed activation antigens CD3+ CD25+ but "Natural Killer" phenotype CD16 or CD56 was always low (< 10%). They had cytotoxic activity against allogenic melanoma cell lines, K 562 (NK activity) and Daudi (NK and LAK activity) cell lines. In 3 patients a specific cytotoxicity against autologous melanoma cell lines was noted.

At the moment, this technique is used to study the efficiency of TIL + IL2 in adjuvant therapy for melanoma stage III. TIL are obtained and injected between 6 and 8 weeks after lymph node resection.

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RESTRICTED USAGE OF GP100-SPECIFIC TCRBVDJ TRANSCRIPTS ASSOCIATED WITH HIGHLY POLYMORPHIC CDR3 REGIONS INDICATES THE LACK OF CLONAL EXPANSION OF TUMOR-REACTIVE TIL IN MULTIPLE GP100-POSITIVE METASTASES OF THREE DIFFERENT MELANOMA PATIENTS. Robert Strohal*, Christine Brna*, Hubert Pehamberger†, Ulrike Mossbacher†, Gottfried Fischer§, Hassane Zarour‡ & Georg Stingl* Dept. Dermatology, *DIAID and †DGD, §Dept. Blood Group Serology, Univ. Vienna Medical School, Vienna, Austria; ‡ Dept Dermatology, Hopital Sainte-Marguerite, Marseille, France.

Controversy exists as to whether anti-tumor immune responses are polyclonal in nature or, alternatively, result from the expansion of clonally restricted TCR specificities targeting the same melanoma-associated antigenic peptides. In order to address this question, we determined TCR usage in tumor-infiltrating lymphocytes of 14 melanoma specimens from three different HLA-typed melanoma patients (HH: nodular melanoma, lymph node, liver, lung, pancreas, brain, skin; GS: liver, lung; SP: lung, spleen, kidney, thyroid gland, skin - HH/SP: HLA-A24, B35, Cw4; GS: HLA-A3, B35, Cw4;) uniformly expressing the melanoma-specific antigens gp100 and Mart-1. Semiquantitative TCRV-specific PCR detected an overall predominance of only one TCRV gene segment family within all three melanoma patients. This is consistent with the hypothesis of a clonally expanded anti-melanoma immune response. However, subcloning and sequencing of the dominating TCRBV specificity revealed that only the primary tumor and a brain metastasis of patient HH harbor T-cells carrying a solitary TCRBVDJ sequence motif, whereas TIL of all other tumor specimens, although preferentially using the same combination of TCRBVJ families, express highly polymorphic CDR3 regions. Together with the identification of a gp100-specific TCRβ-chain motif within skin and lung metastases, our results favor the concept of a tumor-specific immune response against a rather limited number of melanoma-associated antigenic moieties in the patients investigated. We further postulate that this response does not result from expansion and dissemination of only few T-cell clones but rather from the multicentric activation of individual T-cells with different, yet related TCR specificities.

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INTERFERON-γ (IFN-g), IL-12, AND ANTI-IL-10ab AUGMENT THE IMMUNSTIMULATORY POTENTIAL OF B7.1 OR B7.2 TRANSDUCED MELANOMA CELLS

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Preparing a melanoma vacccine of retrovirally transduced human melanoma cells, we have investigated the immunstimulatory capacities of B7.1+ and B7.2+ melanoma cell lines or cell cultures from biopsies (MM).
The cDNAs for human B7.1 and B7.2 were cloned from activated B-cells and inserted in the retroviral vector LXSN. Using this vector amphotropic packaging cell lines were transfected. With supernatants of these cells, MM are transduced and B7+ cells are selected. B7-, B7.1+ and B7.2+ MM (in some experiments preincubated with IFN-g) are coincubated with peripheral blood mononuclear cells (PBMC). Proliferation (in some experiments in the presence of neutralising anti-IL-10 ab, anti-IL-7 ab or IL-12) was assessed by H3 thymidin uptake. After 2 days of coincubation, we screened for the presence of mRNA encoding for IL-2, IL-4, IL-5, IL-10, IL-12p35/40 and IFN-g (RT-PCR) and measured IL-2, IL-4 and IL-10 in the supernatants (ELISAs). Irradiated B7.1+ nor B7.2+ MM did not induce significant proliferation of PBMC. RT-PCR detected IL-10 mRNA, but not mRNA for IL-2 and IFN-g. In the presence of anti-IL-10 ab, no IL-10 mRNA was found, but IL-2 and IFN-g mRNA. IL-2 levels without ab was 0.61 pg/ml , with ab it was 104.54 pg/ml. Similar results were found if coicubation was done after addition of IL-12 or preincubation of MM with IFN-g. Comparing the induced cytokines after stimualtion with B7.1+ and B7.2+ MM, we demonstrate that only B7.2+ can induce IL-4 mRNA.
Our data indicate that the transduction of MM with B7.1 or B7.2 is not sufficient to acheive a significant lymphocytic proliferation with transcription of IL-2 and IFN-g, if IL-10 is present. This problem can be overcome by the preincubation with IFN-g or the addition of IL-12. In addition we showed, that only B7.2+ cells induced IL-4.

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CYTOKINE EXPRESSION IN SPONTANEOUSLY REGRESSING AND NON-REGRESSING PRIMARY MALIGNANT MELANOMAS. Michelle A. Lowes, G. Alex Bishop[2], Kerry Crotty[1], Ross St.C. Barnetson and Gary M. Halliday. Departments of Dermatology and Pathology[1], University of Sydney at Royal Prince Alfred Hospital and Liver Immunobiology Unit, Centenary Institute of Cancer Medicine and Cell Biology[2], Sydney, N.S.W., Australia.

Spontaneous regression is observed clinically and histologically in some primary malignant melanomas (MM). Spontaneous regression, which occurs in the absence of any responsible therapy, is probably immunologically mediated. Regressing MM are infiltrated with larger numbers of activated T cells, primarily CD4+, compared to non-regressing MM. To investigate the hypothesis that spontaneous regression of MM is caused by cytokine production by T cells, cytokine message RNA (mRNA) expression was examined using a non-competitive, quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) method. DNA standards were used to generate known numbers of molecules in each sample. Results were standardised to an internal control, G3PD. CD3δ mRNA was elevated in the regressing group, supporting a role for T cells in regression. TNFβ was significantly elevated in the regressing group. Other Th1 type cytokines, IL-2 and IFNγ, were elevated in the regressing MM. They failed to reach statistical significance, but correlated with CD3δ. The Th2 cytokines IL-10 and IL-13 did not show any difference in the regressing compared to non-regressing MM, neither did the pro-inflammatory cytokines IL-1, 6 and 8 and TNFα. These results indicate that elevated Th1 cytokines, particularly TNFβ, are likely to play a role in spontaneous regression of MM. This supports our hypothesis that activated CD4+ T cells are likely to mediate spontaneous regression of MM by secretion of cytokines.

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EXPRESSION AND EFFECTS OF TRANSFORMING GROWTH FACTOR BETA (TGF-β) ISOFORMS ON PROLIFERATION OF NORMAL MELANOCYTES AND MELANOMA CELL LINES IN VITRO. [1]K. Krasagakis, [2]S. Krüger-Krasagakes, [1]D. Thölke, [1]J. Eberle, [1]M. von der Ohe, [1]C. Garbe, and [1]C.E. Orfanos, [1]Department of Dermatology and [2]Immunology, University Med. Center Benjamin Franklin, Free University of Berlin, Germany.

Previous studies have shown that TGF-β1 may function as autocrine growth inhibitor of melanocytic cells, whereby melanoma cells from metastatic lesions are able to escape from growth control. In the present study, we investigated whether other TGF-β isoforms are involved in melanoma biology. A panel of melanoma cell lines and melanocyte cultures were used as model, and production/secretion levels and inhibitory action of TGF-β2 and -β3 isoforms in comparison to TGF-β1 were studied. RT-PCR analysis revelaled variable mRNA levels of all TGF-β isoforms in both normal melanocytes and melanoma cell lines. Melanoma cell lines secreted higher amounts of TGF-β isoforms than melanocytes as measured by isoform specific ELISAs. No clear-cut differences were observed among melanoma cell lines of primary or metastatic origin regarding expression levels of TGF-β isoforms.TGF-β1 and TGF-β3 were identified as most prominent isoforms, found in increased amounts in supernatants of melanoma cell lines (up to 1.800 pg/ml and 700 pg/ml respectively) and less in melanocytes. TGF-β2 was secreted exclusively by melanoma cell lines (up to 120 pg/ml). Equally strong antiproliferative action of all three TGF-β isoforms was measured in melanocytes using a fluorometric assay (54%, 59%, 56% inhibition at 0.5 ng/ml TGF-β1, -β2 and -β3 respectively). TGF-β1 resistant melanoma cell lines were also TGF-β2 and TGF-β3 unresponsive. The present findings suggest that increased secretion of TGF-β1 and TGF-β3 and new secretion of TGF-β2 are early events in melanoma progression, whereas development of resistance to TGF-β isoforms is a property of metastatic cells from late steps of melanoma progression.

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INFREQUENT HOMOZYGOUS DELETIONS OF THE P16 TUMOR SUPPRESSOR GENE (CDKN2) IN SPORADIC PRIMARY MELANOMA. Thomas Bogenrieder[1], Max Kroiss[1], Anja-Katrin Bosserhoff[1], Josef Rueschoff[2], Ulrich Hohenleutner[1], Michael Landthaler[1], and Wilhelm Stolz[1]. Departments of Dermatology[1] and Pathology[2], University of Regensburg, Germany.

The gene of the cyclin-dependent kinase 4 inhibitor p16 (CDKN2) is a strong candidate for the malignant melanoma (MM) susceptibility gene on chromosome 9p21 and it has been suggested that inactivation of CDKN2 in MM cell lines mainly occurs via homozygous deletion. In contrast, the exact frequency of CDKN2 homozygous deletions in primary MM remains unclear. We therefore examined genomic DNA from 7 benign melanocytic nevi, 10 sporadic primary invasive MM (Clark levels II-IV) and one metastatic MM as well as paired white blood cells (WBC) using comparative multiplex PCR with primers for exon 2 of CDKN2. The chromosome 9q marker, D9S196, served as an internal control.

We identified in 1/10 primary invasive MM and in the metastatic MM but in none of the benign nevi or paired WBC an obvious homozygous deletion of CDKN2. Our data suggest that homozygous deletion is unlikely to be a major mechanism of CDKN2 inactivation in sporadic primary MM, highlighting the need to continue the search for other mechanisms of CDKN2 inactivation (e.g., methylation of 5' CpG islands) and other candidate genes on 9p21 (e.g., p15/CDKN2B).

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INFREQUENT MUTATION OF $p16^{INK4}$ IN SPORADIC PRIMARY CUTANEOUS MELANOMA. Eugene Healy, Stephen Sikkink, Jonathan L. Rees. Department of Dermatology, University of Newcastle upon Tyne, United Kingdom.

Loss of chromosome arm 9p occurs frequently in sporadic cutaneous melanoma, consistent with the involvement of a tumour suppressor gene on this arm in the development of this neoplasm. Mutations / intragenic deletions of the $p16^{INK4}$ gene (located at 9p21) have been identified in several families with inherited melanoma, however, the relative contributions of mutation and deletion in sporadic melanoma is still unclear. We have investigated 26 sporadic cutaneous melanomas (including 14 cases with heterozygous 9p loss) for mutation of $p16^{INK4}$, using a combination of dideoxy-fingerprinting (exons 1 and 3, with subsequent sequencing of cases exhibiting aberrant bands) and cycle sequencing (exon 2).

A $CC \rightarrow TT$ tandem transition (compatible with *in vivo* mutation by ultraviolet radiation) at codons 57 / 58 was identified in one melanoma with 9p loss; this resulted in a premature termination codon, consistent with inactivation of both copies of $p16^{INK4}$ in this case. No mutations were identified in any other melanomas. In addition, no mutations were detected in two Spitz naevi and one benign intradermal naevus with atypical features, all of which had loss of one copy of 9p. Our results suggest that inactivation of $p16^{INK4}$ is important in the pathogenesis of sporadic cutaneous melanoma, but other methods of inactivation of $p16^{INK4}$, such as loss of 9p, rather than point mutation may be more relevant. The low mutation frequency of $p16^{INK4}$ in our cases with heterozygous 9p loss is also consistent with another tumor suppressor gene on 9p being involved in some cases of sporadic melanoma.

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VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) EXPRESSION IN SERA AND LESIONS OF PATIENTS WITH MALIGNANT MELANOMA. A Claudy, J.Viac, D.Schmitt.Clinique. Dermatologique & INSERM U346, Hôpital Ed. Herriot 69437 Lyon 03 France

Among angiogenic peptides,VEGF, is expressed and secreted by several kinds of cells including tumour cells. In order to determine whether VEGF could be involved in the clinical course of malignant melanoma, we studied 78 patients with primary or metastatic melanoma. The follow-up of 9 patients, previously selected, was also included because they initially presented with a primary melanoma and further developed metastasis over a period of 12 to 25 months. Circulating VEGF levels were quantified by enzyme-linked immunosorbent assay (R&D Systems) and the reactivity patterns of VEGF in cutaneous melanoma was assessed using two anti VEGF antibodies (R&D Systems; Santa Cruz Biotechnology). Significantly elevated levels of VEGF occurred in sera of patients with primary or metastatic melanoma compared to a control group ($p<0.001$). Immunohistochemical stainings of primary cutaneous melanoma, showed a lack or a discrete VEGF reactivity of melanoma cells according to their location in the epidermis or in the dermis. Endothelial cells, some inflammatory cells and activated keratinocytes were strongly stained. These results suggest that increased VEGF levels in patients' sera likely contribute to the development of metastasis and may represent an additional indicator of poor prognosis in malignant melanoma.

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ALTERED SIALYLATION OF PNA-BINDING GLYCOPROTEINS REFLECTS INVASIVENESS OF HUMAN MELANOMA CELLS. O. Berthier-Vergnes[1], P. Broquet[2], P. Delannoy[3], N. Zebda[4], M. Gaucherand[1], D. Schmitt[1]. **1** INSERM U346, Hôpital E. Herriot, 69437 Lyon; **2** INSERM U189, Oullins, France; **3** UMR CNRS 111, Villeneuve d'Ascq; **4** New York University Medical Center, NY 10016.

Aberrant glycosylation of tumor antigens is associated with the metastatic process. We have shown that Peanut Agglutinin (PNA) detects tumor cells on primary melanomas, dependent of depth and thickness (1). Normal melanocytes in the skin or cultured without promoters are not stained by PNA as non metastatic melanoma cells (2). Using a metastasis model, we showed that melanoma cells selected for their high affinity sites for PNA generate lung metastases. These metastasizing cells exhibit a deficiency in sialylation of terminal galactose shared by a) 2 O-glycosylproteins of 140 and 110 kDa b) lactosylceramide (2, 3). Sialyltransferases (ST) are a family of enzymes which transfer sialic acid (SA) to terminal position of the oligosaccharide chains of glycoproteins and glycolipids. These enzymes differ in their specificity towards different acceptors and in how they link SA to the penultimate sugar. We have shown that metastatic cells have an impaired ganglioside biosynthesis due to a low activity of the α-2,3 ST acting on lactosylceramide (3). In this study, we investigate ST responsible for sialylation of O-glycans linked to proteins. Results showed that metastatic cells exhibited a 2 fold higher activity of α-2,3 ST as compared to non metastatic cells, and similar activities of α-2,6-ST. However, they express comparable amounts of cell surface SA and bind SNA and MAA with the same intensity. Endogeneous sialidase activity was equivalent in both cells. Our data suggest that unsialylated Galß1-3GalNac on metastatic human melanoma cells is due to a downregulation of α-2-3 ST. Whether regulation of α-2-3 ST is at the transcriptional or at the enzymatic levels is under investigation.

1 Berthier et al., Lancet, 1993, *341*, 1292; **2** Zebda et al., J Cell Biochem, 1994, *54*, 161; **3** Zebda et al, FEBS Letters, 1995, *362*, 161.

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EXPRESSION OF THE VITRONECTIN RECEPTOR αvß3 INHIBITS INVASION AND METASTASIS OF HUMAN MELANOMA CELLS.

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From several studies it is known that expression of integrin αvß3 emerges during melanocytic tumor progression and has been implicated in growth and invasion of human melanoma cells. Conversily, it has also been shown that downmodulation of αvß3 can enhance the invasive potential of certain melanoma cells.

To study the role of αvß3 in melanoma cell invasion and metastasis we transfected ß3 cDNA into the highly metastatic, ß3-negative, human melanoma cell line MV3. As a concequence of this transfection we found:

1. Marked αvß3 expression on the cell surface.
2. αvß3-mediated activation of α5ß1.
3. Induced adhesion to fibrinogen, which is both αvß3 and α5ß1 mediated.
4. Reduced invasion in in vitro invasion assays.
5. Complete inhibition of experimental metastasis after intravenous inoculation into nude mice.

These results show that induction of αvß3 affects adhesion, interfers with invasion and inhibits metastasis of MV3 melanoma cells. We conclude that αvß3 either can facilitate or inhibit metastasis formation of melanoma cells, dependent on its cellular background.

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IMMUNOREACTIVITY OF GLYCOPROTEIN IIB IS PRESENT IN METASTASIZED BUT NOT IN NON-METASTASIZED PRIMARY CUTANEOUS MALIGNANT MELANOMA

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Cell adhesion molecules play an important role in metastasizing malignant melanomas. It has been shown that melanoma cells bearing the fibrinogen-(αIIbβ3) or the vitronectin-receptor (αvβ3) are highly adhesive with platelets.

This study evaluates the presence of platelet integrin αIIbβ3 subunits on cells of primary cutaneous malignant melanomas with (n=10) and without (n=10) metastases over a time period of six years. Metastases of melanomas were not investigated.

Monoclonal antibodies directed against the subunits of αIIbβ3 receptor were used on paraffin-embedded sections. Because of a possible cross-reactivity of αIIbβ3 with αvβ3, a monoclonal antibody against the alfa-chain of the vitronection receptor (αv) was used. Staining was performed by means of immunohistochemistry using the alkaline phospatase method.

The subunit αIIb was exclusively present on cells of metastatic but not on non-metastatic melanomas. The subunits β3 and αv were found to stain sections from all used tissues.

These data favour the role of the integrin receptor αIIbβ3 in the metastatic behaviour of malignant melanomas.

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UNIVERSAL KERATIN EXPRESSIONS AND A NOVEL PROTEIN IN FIVE CULTURED MELANOMA CELL LINES DERIVED FROM PRIMARY, RECURRED, AND METASTATIC MELANOMAS.

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Up to now, it has been noted that keratin, one of intermediate filaments, may not be expressed in cultured human melanoma cells. However, using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) we have demonstrated in this paper that keratin polypeptide expressed in five kinds of established culture cell lines derived from primary, recurred, and metastatic melanomas. Combined with the data of our previous paper (*J. Dermatol. Sci.*, (1996) in press), expression of keratin polypeptide was considered universal evidence for establishing melanoma cell lines in vitro. In addition, we detected two particular polypeptides (Mr, Ca. 80 Kd, pI 7.8; Mr, Ca. 48 kd, pI 8.2) that do not correspond with the reasonable positions of any keratin polypeptides on 2D-PAGE in all employed cell lines. From the results of a BLAST homology search, the latter polypeptide was human ATP synthase α-chain, which was also expressed in five kinds of cultured SCC and eight other types of melanoma cell lines, described in our previous paper. On the other hand, the former was expressed only in the five cell lines employed in this paper and did not conform with any polypeptides in the above homology search. We propose that this polypeptide may be a novel protein in cultured melanoma cell lines and have it a melanoma-related protein.

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MALIGNANT MELANOMA CELLS EXHIBIT DIFFERENCES IN VITAMIN A METABOLISM AND mRNA-EXPRESSION OF RETINOID BINDING PROTEIN AND RECEPTOR GENES AS COMPARED TO HUMAN MELANOCYTES.

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Retinoids inhibit proliferation of cultured melanocytes and melanoma cells and affect disorders of hypo- and hyperpigmentation. Such effects might involve retinoid binding proteins, activated retinoid metabolites and nuclear retinoid receptors for transcriptional activation. We detected mRNA transcripts for the cellular retinol- and retinoic acid-binding proteins (CRBP, CRABPI and II) in cultured epidermal melanocytes. In the melanoma cell lines the major transcript was CRABPII. The mRNA level was not reflected at the protein levels of CRBP or CRABPII, which were similar in the melanocytes and melanoma cells. Nuclear retinoic acid receptor (RARα, RARβ, RARγ, RXRα) transcripts were detected in all cells. The amount of transcripts for RARβ was higher in melanoma cells than in melanocytes. The endogenous concentration of retinol (ROH) and its metabolite 3,4-didehydroretinol (ddROH) in melanocytes were 5-fold that in melanoma cells. When incubated for 24 hours with [^{3}H]ROH the main metabolites in the melanocytes were [^{3}H]ddROH (4%), [^{3}H]RA (0.4%), and [^{3}H]13-cis-RA (≤0.1%). Formation of [^{3}H]RA was only detected in one melanoma cells line. When adding [^{3}H]RA we found the major metabolite to be [^{3}H]13-cis-RA. Both melanocytes and melanoma cells produced a yet unidentified metabolite when incubated with [^{3}H]ROH and [^{3}H]RA. Aberrant expressions of CRBP, CRABPI and RARβ, dissimilarities in metabolism and endogenous concentration of retinoids between benign and malignant melanocytes might play key roles in the modulation of differentiation and growth.

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MOLECULAR ABNORMALITIES IN MTS1 GENE. STUDY IN SPORADIC MALIGNANT MELANOMA.

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The MTS1 gene, located on short arm of chromosome 9, seems to be involved in the development of various types of cancers, especially in familial melanoma. The aim of our study was to evaluate the involvement of this anti-oncogene in sporadic melanoma. We focused our research on the loss of allele in the 9p21 region and on mutations on exon 2.

PATIENTS & METHODS: 20 samples of melanoma tissue were obtained from 19 patients, including one familial melanoma, with a mean age of 62. Samples were 14 metastatic cutaneous lesions, 4 primary tumors and 2 lymph nodes. Histology confirmed melanoma on all samples. Mean Breslow thickness of primary tumors was 4,79 mm. DNA was extracted from tumors and peripheric blood with a technique using K-proteinase and phenol-chloroform. Loss of allele was performed by genic amplification of micro-satellites sequences (D9S171 and D9S265) located on 9p21. Mutation were screened with denaturing gradient gel electrophoresis followed by direct sequencing.

RESULTS & DISCUSSION: Nowadays, 9 samples have been analysed. Loss of allele was recognized on 2/4 informative cases. The analysis performed on 75% of the coding sequence of exon 2, revealed no abnormalities. These preliminary results, obtained on a short serie, showed that in 50% of informative tumors, we observed a loss of allele, indicating presence of an anti-oncogene in the 9p21 region. The lack of mutation on the part of the gene studied, let us assume that abnormalities of MTS1 are located on exon 1 or 3 or that an other anti-oncogene (MTS2 ?) present in this area could be involved in this type of tumor.

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FREQUENCY OF DIFFERENT TUMORS IN ITALIAN MELANOMA-PRONE FAMILIES WITH THE MUTATION OF p16 (Gly93Trp).

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The aim of our study is to verify the frequency of different tumors in Italian Melanoma-prone families with a p16 mutation. Previous studies indicate that p16 (CDKN2) mutations are required for pancreatic cancer development in 10 9p21 linked melanoma-prone kindred. Evidence relating an increased risk of pancreatic adenocarcinoma to the presence of a p16 mutation (p16 M) has been found by us in bloodline members of Italian-melanoma-prone families. Thus far, we have detected the same Gly93Trp mutation in 7 apparently unrelated families, and in none of the 50 control individuals - all within the same small geographic area of Italy (possibly due to "founder effect"). Nineteen melanoma and three dysplastic naevus cases, were diagnosed at ages ranging from 21 to 70 in the kindred harboring the mutation. In addition, we have found 15 cases of cancer at other sites in these kindred. We found 4 pancreatic cancers out of 80 members (except spouses) in our 7 p16M families vs 0.43 expected [P (Fisher)<10^{-5}] and 4 breast cancers out of 35 members (except spouses) in 7 p16 Wild-type (p16W) families vs 1,04 expected [P (Fisher)=0.02].

In contrast, 18 melanoma and 4 dysplastic naevus cases and 9 cases at other sites, including 4 stomach cancers but no pancreatic cancers, were detected in 7 melanoma-prone p16W. The data we have obtained support the hypothesis that there exists an association of a pancreatic cancer risk with the impairment of the p16 function, and suggests a possible specific role of the CDKN2 in tumorigenesis.

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SCREENING FOR MELANOMA. NEW ASPECTS. MJM de Rooij[1], FHJ Rampen[2], LJ Schouten[3], HAM Neumann[4]. [1]University Hospital St Radboud, Nijmegen; [2]St Anna Hospital, Oss; [3]IKL Maastricht and IKO Nijmegen; [4]University Hospital Maastricht, the Netherlands.

Annual, voluntary, skin cancer and melanoma screening clinics have been held in the United States since 1985. Similar screenings have been carried out as pilot studies since 1989 in the Netherlands. The first campaigns concerned melanoma and skin cancer in general. The present study was focused on malignant melanoma only. In total 4146 participants were screened on two consecutive Saturdays in June 1993. Out of these persons, 486 (11.7%) had a suspicious premalignant or malignant lesion and received a letter of referral for his or her general physician indicating the proposed line of management. Participants with borderline lesions or minimal extent of precursor states were not referred; referral of all these persons should have resulted in almost a doubling of the number of referred cases (22%). All referred screenees but two gave permission for follow up. Adequate follow up was accomplished in 99%. Only 4 screenees were lost during follow up (clinical diagnoses: basal cell carcinoma 1, congenital naevus 1 and dysplastic naevi 2). Compliance with referral was nearly complete. Only 1 screenee with a presumed basal cell carcinoma and 6 screenees suspicious of having a premalignancy (dysplastic naevi 5 and congenital naevus 1) decided not to seek medical attention despite several reminders. Totally, 13 melanomas (all but 1 with Breslow thickness < 1mm), 43 basal cell carcinomas, and 1 Bowen's disease were histologically confirmed. It is concluded that a selective referral policy (no borderline cases) reduces the generated costs of skin cancer/melanoma screenings considerably and prevents unnecessary medicalisation. Adequate follow-up of positive screenees is extremely important in order to determine the ultimate yield of such campaigns.

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TUMOR-STROMA INTERACTION IN BENIGN AND MALIGNANT MELANOCYTIC SKIN LESIONS

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In recent years numerous studies pointed out, that tumor invasion and metastasis are complex processes based on interactions of the tumor cells and the surrounding stroma. In the present study we investigated certain morphological criteria of tumor-stroma interaction in benign common nevi, Spitz's nevi, primary malignant melanoma and melanoma metastatic to the skin. In particular, three morphological features of tumor-stroma interaction were assessed in 278 melanocytic nevi, 322 Spitz's nevi, 368 cases of primary malignant melanoma and 344 cases of melanoma metastatic to the skin, respectively. DERMSIMPLE was defined as simple infiltration of melanoma cell nests, strands or single cells between collagen bundles of the reticular dermis without evident inflammatory cell infiltrate or fibroplastic stroma reaction, PRECOLL expressed the existence of morphologically intact collagen bundles of the reticular dermis within the tumor bulk and PREFAT expressed the existence of morphologically intact single fat cells within the tumor bulk.

The feature DERMSIMPLE was found in 53 (19%) benign common nevi, in 35 (11%) Spitz's nevi, in 72 (45%) cases of primary malignant melanoma, and in 129 (38%) cases of melanoma metastatic to the skin (Goodman & Kruskal Gamma test; p=0.00001). PRECOLL was expressed in 47 (17%) cases of melanocytic nevi, in 74 (23%) Spitz's nevi, in 109 (30%) cases of primary malignant melanoma and in 155 (45%) cases of melanoma metastatic to the skin (p=0.00001). The feature PREFAT was found in 7 (3%) benign nevi, in 4 (1%) Spitz's nevi, in 16 (4%) cases of primary malignant melanoma and in 106 (31%) cases of melanoma metastatic to the skin, respectively (p=0.00001).

The study shows that simple infiltration into the surrounding stroma without morphological evidence of stroma destruction or reactive proliferation increases with tumor progression.

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AUTOMATIC MELANOMA SECTION AREA MEASUREMENT PROVIDES PROGNOSTIC INFORMATION.

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It has been suggested that tumor volume is an essential three-dimensional prognostic feature of cutaneous melanoma. The study addresses the question whether measurement of melanoma section area as a two-dimensional feature is a better prognostic indicator than one-dimensional tumor thickness alone.

From 90 cases of primary melanomas of the skin the H&E section showing the largest vertical tumor thickness was selected. At 100 x magnification, the entire section was automatically scanned and the area covered by tumor was recorded by a user-independent image analysis procedure and given as mm².

Melanoma section area ranged from 0.02 to 83.50 mm² (median 3.42) and showed a significant correlation with vertical tumor thickness ($r = 0.79$, $p < 0.001$). Overall 3-year survival was 73 +- 11 % in cases with an area > 10 mm² compared to 100 % in cases with an area < 10 ($p < 0.0001$). Multivariate analysis using Cox' model revealed area as a superior prognostic parameter ($p = 0.004$) compared to tumor thickness ($p = 0.006$).

The study shows that user-independent automated image analysis with whole section scanning provides prognostic information from routine H&E sections beyond that obtained by tumor thickness alone.

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Expression of nm23 protein in acquired melanocytic nevi, malignant melanoma and metastases of malignant melanoma: an immunohistological assessment in human skin. P. Seyfried[1], R. Braun[1], B. Berger[1], F.A. Bahmer[2], J. Reichrath[1]. [1]Dept. of Dermatology, Universität des Saarlandes, Homburg/Saar; [2]Dept. of Dermatology, Zentralkrankenhaus St. Jürgen Str., Bremen, Germany.

Expression of nm23-HI gene mRNA has been shown *in vitro* and *in vivo* to correlate inversely with metastatic potential in various human tumours, including malignant melanoma and breast cancer. We have characterized immunohistochemically *in situ* expression of nm23 polypeptide in acquired melanocytic nevi (common acquired nevi, n = 19; dysplastic nevi, n = 19), malignant melanomas (n = 22), and metastases of malignant melanomas (n = 47). Analyzing paraffin sections, we found heterogeneous expression of nm23 protein in varying intensities in all analyzed tumours of human skin, predominantly in cytoplasmic cell compartments. Additionally, nuclei and cell membranes of single scattered melanocytic cells were labeled as well. Comparing immunostaining for nm23 polypeptide, no correlation of nm23 expression with malignant phenotype was observed. No visuable differences in labelling intensity or staining pattern of nm23 polypeptide were found comparing benign acquired melanocytic nevi, malignant melanomas, and metastases of malignant melanomas. In conclusion, our results indicate that (i) nm23 polypeptide is predominantly expressed in the cytoplasmic but also in nuclear and membrane compartments of melanocytic human cells, (ii) expression of nm23 protein does not correlate with benignant or malignant phenotype in melanocytic tumours of human skin, and (iii) expression of nm23 polypeptide is not inversely correlated to the metastatic potential of metastases of malignant melanomas.

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ALLELIC DELETION DETECTED ON CHROMOSOME 1p AND CHROMOSOME 9p IN MICRODISSECTED DYSPLASTIC NEVUS LESIONS

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Genetic changes which contribute to carcinogenesis include activation of oncogenes and inactivation of tumor suppressor genes. Frequent allelic loss at specific loci in tumor cells implies functional inactivation of a putative tumor suppressor gene. Very little is currently known of the molecular events associated with transformation of dysplastic nevi to cutaneous melanoma, even though they represent clinical and histopathologic precursors of cutaneous melanoma. Previous studies showed that inactivation of tumor suppressor genes on chromosomes 1 and 9 are associated with melanoma.

The aim of this study was to test for loss of heterozygosity (LOH) on different loci on chromosomes 1 and 9 on paraffin-embedded suspect areas in dysplastic nevi.

Clusters of melanocytes of 9 archival dysplastic nevi as well as the adjacent tissue were procured under direct microscopic visualization using the microdissection technique, followed by a single-step DNA extraction. Extracted genomic DNA was amplified by PCR using three polymorphic markers on chromosome 1 (D1S243, D1S450, D1S1646) and one marker on chromosome 9 (D9S12).

Of 9 dysplastic nevi, 4 (47%) showed allelic deletion either on chromosome 1p (3/9) or 9p (1/6). In one case, two separate dysplastic nevi were selected; one of them showed LOH on 1p whereas the other showed no allelic deletion.

Allelic deletion can be detected in microdissected dysplastic nevi. Our results show that dysplastic nevi appear to have at least two discrete areas of deletion either on chromosome 1p and/or 9p, suggesting that different genetic events may contribute to dysplastic nevus formation. The high incidence of allelic LOH in selected areas of chromosomes 1 and 9 in dysplastic nevi strongly suggest that dysplastic nevi truly represent precursors of melanoma. Therefore, the presence of tumor suppressor genes on chromosome 1 and 9 may play a role in the development of dysplastic nevus and progression into melanoma.

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RISK OF CUTANEOUS MALIGNANT MELANOMA IN PATIENTS WITH NON-FAMILIAL DYSPLASTIC NEVI. D.G.C.T.M. Snels, W. Bergman, N.A.Gruis. Department Dermatology, University Hospital Leiden, The Netherlands.

Dysplastic or atypical nevi (AN) are regarded as one of the major risk factors for the development of malignant melanoma of the skin (CMM). AN occurring within certain melanoma prone (FAMMM) families are markers of a life-time melanoma risk of about 100%, however this FAMMM syndrome is rare. AN are also known to occur in individuals in whom a family history of melanoma is lacking. This is named the sporadic atypical mole (SAM) syndrome. Depending on the definitions used this SAM syndrome is seen quite frequently (2.4-28%). Recently the first risk calculations in individuals with SAM syndrome have been reported to vary between 15-92. We have conducted a study including 166 persons with SAM syndrome, yielding a total of 925 personyears of follow-up. During these 925 personyears three invasive CMM were diagnosed and two melanomas in situ. Regarding only the three invasive CMM a relative risk of 36 (CI 6.5-91) was calculated as compared to the general Dutch population. One of our further aims was to try to verify a "dose-dependent" risk, by considering the total number of AN in each person in relation to the risk of melanoma. However, one invasive CMM happened to occur in a person with only 5 AN and one melanoma in situ occurred in a person with only 9 AN making a statistical analysis almost redundant. Further studies are needed including hundreds of patients which will facilitate a detailed study of several phenotypical variations of SAM syndrome in relation to melanoma risk.

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A SINGLE ERYTHEMAGENIC UV-IRRADIATION IS MORE EFFECTIVE IN ENHANCING THE PROLIFERATIVE/REPARATIVE ACTIVITY OF MELANOCYTES IN MELANOCYTIC NEVI COMPARED TO FRACTIONALLY APPLIED HIGH DOSES.

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In order to study the effect of UV-light on the expression of HMB-45, Ki 67, topoisomerase IIa, PCNA, and p53 in melanocytic nevi in vivo, we immunohistochemically investigated nevi after a single erythemagenic UV-dose (2 MED and 4 MED UVB) and after daily therapeutical UV-exposures over 4-6 weeks. To compare UV-induced changes of the non-irradiated part of the lesion, one half of the nevus was covered by a black tape during irradiation. Except for the antibody against HMB-45, a double staining procedure using anti-Vimentin as the other primary antibody was applied to be able to distinguish between labelling in keratinocytes and melanocytes. In low-dose irradiated nevi (n=10) we observed only slight differences in the UV-irradiated part compared to the non-irradiated part of the same nevus. One week after the single UV-exposure (n=11) in melanocytes of the irradiated part expression was enhanced of HMB-45 in 8, Ki 67 in 8, topoisomerase IIa in 7, PCNA in 7 of the investigated cases. The keratinocytes showed an increased labelling for PCNA and p53 in the irradiated part of the lesion, whereas the p53-expression in melanocytes was not significantly altered after UV-exposure. Compared to repeated low-dose UV-exposure a single erythemagenic dose of UV-light is much more effective in the induction of melanocytic proliferation in nevi in vivo. These findings support the importance of intermittent UV-exposure in the pathogenesis of pigmented lesions.

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TWO MINIMAL ERYTHEMA DOSE OF UV RADIATION CAN INDUCE CHANGES IN DERMOSCOPIC FEATURES OF ACQUIRED MELANOCYTIC NEVI.

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Several studies have indicated that UV radiation can lead in long term and in short term to clinical, histopathological, and ultrastructural changes in melanocytic nevi. In this study, we investigated whether radiation with two minimal erythema dose (MED) can lead to changes of melanocytic nevi detectable by digital dermoscopy.

Seven acquired melanocytic nevi of two volunteers were exposed to two MED of a Sellasol lamp with an mean intensity of 7.8 mJ/cm^2 in the UVB range and an mean intensity of 87.8 mJ/cm^2 in the UVA range. Digital dermoscopic images taken before and seven days after radiation were viewed side by side on the screen of the digital dermoscopic workstation by two different investigators. Ten different parameters describing symmetry, border, color, pigment network structures, and pigmented globules of the nevi were scored using a visual analogue scale from 0 to 10.

After radiation the border of nevi became more faded (matched pairs signed rank test: $p<0.05$). Nevi also became darker brown ($p<0.05$) and hypopigmented areas appeared to be smaller. In addition, the pigment network became more irregular and more broader faded. Pigmented globules also increased after UV radiation.

We conclude that radiation of acquired nevi with two MED can induce changes that can be visualized by digital dermoscopy. These findings suggest that UV doses inducing erythema may also lead to activation and eventually proliferation of melanocytes within nevi.

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LASER TREATMENT OF COMMON AND ATYPICAL MELANOCYTIC NEVI.

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Background: Clinical and histological atypic or dysplastic nevi are known for their increased potential for malignant degeneration and are often regarded as cosmetically disturbing. Q-switched laser has become a major mode of treatment for cutaneous pigmented lesions without scarring.

Objective: To assess the clinical and histological effects of Q-switched laser in small common and atypical melanocytic nevi.

Methods: 50 common nevi and 50 dysplastic nevi were selected. Each lesion was photographed and the color was measured using a CR 300 Minolta Chromameter. The lesions were treated with a Q-switched Alexandrite laser (AlexLAZR, Candela Laser Corp.) with a 755 nm wavelength, 50 nsec pulse duration at a 8 J/cm^2 fluence. After 3 treatments with 2 month intervals, the lesion was excised and histologically examined. In a number of lesions only 50% was treated, the untreated 50% acting as internal control.

Results: The first results show a marked clinical reduction of pigmentation after 3 laser treatments in macular, heavily pigmented lesions. Lightbrown and slightly papular lesions showed a very poor response. No significant differences in response could be observed between the common and atypical nevi. In a majority of cases a marked hypopigmentation could be observed. Histopathology showed nevus cel reduction rates matching clinical resolution.

Conclusions: Q-switched laser is an effective treatment for heavily pigmented macular melanocytic nevi, but not for papular and lightbrown nevi. The possible effects of sublethal doses of laser on cellular processes are currently under investigation.

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ENGRAFTMENT OF HUMAN BASAL CELL CARCINOMA IN HUMAN SKIN GRAFTED TO IMMUNODEFICIENT MICE.

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Experimentation with one of the most common human tumors, basal cell carcinoma (bcc) is very difficult. Therefore *in vitro* cell culture methods have not become routinely applied as in the case of melanoma or squamous cell cancer. Because previous efforts to create *in vivo* models for bcc were hampered by low tumor take, we attempted to create and characterize a new model allowing *in vivo* experiments. To improve take-rate by creating a tissue-specific human microenvironment, we grafted bcc into human skin previously transplanted to mice with Severe Combined Immunodeficiency (SCID mice).

SCID mice were transplanted with healthy full thickness human skin. When the grafts healed in completely, small slots were made in the human skin graft, into which 1-2 mm^3 tumor fragments were inserted and sewn. After 8- to 40 weeks post engraftment the growing tumors were removed, routine light microscopy, immunohistochemistry and/or electron microscopy were performed.

Twelve bcc grafts were prepared that have uniformly shown presence of a small, either stabile or slow growing tumor nodule at four to twenty weeks post-op. Investigations of the biopsies revealed that the transplanted nodular or pigmented tumors preserved their morphological characteristics.

It is possible with the described new *in vivo* tumor model to evaluate new therapeutic approaches, and to study the biology of human bcc more profoundly than in the past.

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SOLID BASAL CELL CARCINOMA PROBABLY ORIGINATES FROM THE OUTER ROOT SHEATH OF THE VELLUS HAIR FOLLICLE.

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The origin of basal cell carcinoma (BCC) is still under discussion. The aim of this study was to investigate the immunophenotype of solid BCC and to compare it to the vellus hair follicle (vellus HF) and normal epidermis of the face.

Biopsies were processed for immunohistochemistry and the presence and distribution of a wide panel of epithelial cytokeratins (CK 1, 4 , 5, 7, 8, 10, 13, 14 and 19) and trichohyaline has been analyzed using monoclonal antibodies. Also the lectin binding capacity was investigated using lectins with different sugar specifities (UEA-1, SBA, DBA, PNA, Con A, WGA).

BCC cells and the outer root sheath keratinocytes of the vellus HF showed almost identical immunohistochemical profiles for cytokeratins and trichohyaline and for lectins. In particular, we found a corresponding expression of the basal differentiation markers CK 5 and CK 14 in BCC, in the outer root sheath of the vellus HF and in the basal cell layer of the epidermis. In contrast neither the suprabasal differentiation markers CK 1 and 10 nor the simple epithelium type keratins 7 and 8 or the inner root sheath markers CK 13 and trichohyaline could be demonstrated. Interestingly, cytokeratin 19, a simple epithelium marker associated with premalignancies, showed constant expression in the outer root sheath of the vellus HF with pronounciation in the bulge area, the presumptive follicular stem cell region. On the other hand CK 19 was expressed in the majority of BCCs, while it was always negative in the epidermis. Lectin binding profiles in BCC, in the outer root sheath of the vellus hair follicle and in basal epidermal cell layers were similar presenting binding sites for the lectins PNA, Con A and WGA.

In conclusion, these immunohistochemical findings underscore a possible origin of BCC from the outer cells of the outer root sheath of the vellus hair follicle and/or the bulge region.

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BASAL CELL BUT NOT SQUAMOUS CELL CARCINOMAS OF THE SKIN EXPRESS THE NON-METASTASIS NM23 GENE.

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The nm23 gene encodes for a 17 kDa protein, identical with NADP kinase; it is believed to exert a metastasis-suppressor function since its expression is reduced in some metastatic solid malignancies. Because very few data exist on the role of nm23 in cutaneous neoplasms, we studied the immunohistochemical expression of the nm23 gene product in frozen sections of normal skin and of 89 various cutaneous tumours, by using a specific monoclonal antibody (37.6) to nm23. Nm23 was found expressed within basal cells of the epidermis and its appendages. All basal cell carcinomas (BCC, n:23) showed diffuse immunoreactivity; by contrast, only one out of eleven squamous cell carcinomas (SCC) showed substantial immunoreactivity. Premalignant lesions (Keratoses, Bowen's Disease) (n:25) and the benign epithelial lesions studied (n:9) showed weak immunoreactivity, confined to basal cells. Benign Naevi (n:4) and most (9/12) Malignant Melanomas expressed nm23 immunoreactivity and the pattern observed was similar between primary (n:3) and metastatic (n:9) lesions. Kaposi's sarcoma lesions (n:4) were unreactive whereas the case of Malignant Fibrous Histiocytoma disclosed 30% of nm23-positive cells. These results show that nm23 is differentially expressed in cutaneous tumours. It seems likely that the strong nm23 immunoreactivity of BCC, contrasting with the absent expression in SCC, reflects the different metastatic potential of these two tumour types. The nm23 gene seems to be involved in cutaneous carcinogenesis; its role may be different according to the histogenetic type of tumour, and clearly deserves further study.

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EXPRESSION OF RETINOIC ACID RECEPTOR PROTEINS IN BASAL CELL CARCINOMAS: AN IMMUNOHISTOCHEMICAL ANALYSIS

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We have analyzed immunohistochemically the expression of RAR-α,-β,-γ proteins in basal cell carcinomas (BCCs, n = 9) *in situ*. Labelling pattern for the different types of RARs was compared with staining pattern of the proliferation marker Ki-67 in the same tumours. We found strong immunoreactivity for RAR-α and RAR-γ in all BCCs analyzed, while no or very weak staining for RAR-β protein was detected. Staining of BCCs for RAR-α was pronounced as compared to staining for RAR-γ. In contrast to RAR-γ, that revealed no or only marginal differences in staining intensities, RAR-α immunoreactivity was consistently stronger in BCCs as compared to adjacent unaffected epidermis. In general, labelling of BCCs for RAR-α and RAR-γ was pronounced in cells of the palisade and peripheral cells, while staining in the center of the tumours was heterogenous. The majority of BCCs revealed no visual correlation comparing labelling patterns for RAR-α and RAR-γ with labelling pattern for Ki-67, while in a few specimens expression of RAR-α, RAR-γ and Ki-67 proteins was confined to peripheral tumour cells. Our findings indicate that (I) RAR-α and RAR-γ are in contrast to RAR-β strongly expressed in BCCs (II) expression of RAR-α seems to be upregulated in BCCs as compared to keratinocytes of uninvolved epidermis (III) BCCs may be targets for potentially preventive or therapeutical treatment with RAR-α- or RAR-γ-selective retinoic acid metabolites.

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DECREASED EXPRESSION OF INTERFERON-GAMMA RECEPTORS AND INTERFERON-GAMMA INDUCED UPREGULATION OF INTERCELLULAR ADHESION MOLECULE-1 IN BASAL CELL CARCINOMA.

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Basal cell carcinoma (BCC) is the most common skin cancer in humans. It is a slowly growing malignant epithelial tumor. Although seldom metastatic, it causes severe local destruction and disfigurement if left untreated. The local defence against BCC comprises of peritumoral inflammatory infiltrate which consists mainly of T lymphocytes with negligible participation of B cells and Natural Killer (NK) cells. The ability of immune effector cells to recognize and adhere to tumor cells is an imperative first step in cell-mediated cytotoxicity. Intercellular adhesion molecule-1 (ICAM-1) plays a pivotal role in T lymphocyte adhesion and cytolytic activity. Interaction of LFA-1 on the T cell and ICAM-1 on the target cell is necessary for T cell proliferation. The reported absence of ICAM-1 on BCC cells may explain the lack of active immunity against this tumor. In the present study, the expression of IFN-γR and the induction of ICAM-1 was investigated in short-term cultures of BCC biopsies using recombinant human IFN-γ. The results showed that stimulation with rHuIFN-γ led to significantly increased expression of ICAM-1 on the tumor cells and to a significant increased shedding of ICAM-1 in the culture supernatants. The expression of IFN-γR was significantly decreased on the tumor cells as compared with the overlying epidermis. The decreased expression of IFN-γR on the tumor cells and the shedding of ICAM-1 into the surrounding stroma may protect the tumor cells against an active immune response.

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COLLAGENASE-3 (MMP-13) IS EXPRESSED BY SQUAMOUS CELL CARCINOMAS OF HEAD AND NECK.

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Collagenase-3 (MMP-13) is a novel matrix metalloproteinase, the expression of which has so far been detected only in human breast carcinoma tissue and osteoarthritic cartilage. We have generated a 1.6 kb human MMP-13 cDNA with RT-PCR and examined expression of MMP-13 mRNA in 21 cell lines established from primary squamous cell carcinomas (SCCs) of head and neck area and in 7 cell lines from metastases of SCCs. Expression of MMP-13 was detected in 13 out of 21 primary SCC lines and in 5 out of 7 SCC metastasis cell lines. In comparison, interstitial collagenase (MMP-1) mRNA was expressed by 10 out of 21 primary SCC cell lines and by 4 out of 7 cell lines of SCC metastases. Interestingly, only 4 primary SCC lines and 1 metastasis cell line were negative for both MMP-13 and MMP-1 mRNA. Expression of MMP-13 mRNAs was also detected in human cutaneous SCCs using *in situ* hybridization. In contrast, no expression of MMP-13 mRNAs was detected in normal human epidermal keratinocytes in culture or in intact adult human skin. Our data show that MMP-13 is expressed by SCC cells *in vitro* and *in vivo*. The lack of MMP-13 mRNAs in normal human epidermal keratinocytes *in vitro* and *in vivo* indicates that MMP-13 expression is confined to epidermal keratinocytes which have undergone malignant transformation.

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EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN EPITHELIAL TUMOURS IS RELATED TO KERATINOCYTE ACTIVATION AND DIFFERENTIATION.

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The expression of VEGF was investigated in normal skin, benign and malignant epithelial tumours and cultured keratinocytes in order to correlate VEGF expression with the proliferative activity and degree of differentiation of keratinocytes. Skin lesions were tested by immunohistochemical stainings using two anti-VEGF antibodies. Secretion and production of VEGF by keratinocyte cultures were evaluated by enzyme-linked immunosorbent assay (R&D Systems). Low to moderate VEGF expression was observed in normal epidermis. In epithelial tumours, different reactivity patterns were observed and different areas of the same tumour expressed variable amounts of VEGF. A more prominent labelling may occur either in proliferative layers or/ and in more differentiated cells of viral-induced lesions, squamous cell carcinomas and Bowen's disease whereas basal cell carcinomas always stained weakly for VEGF. In cultured keratinocytes, the amount of cell-associated and secreted VEGF increased with time and the constitutively produced VEGF was mostly released extracellularly. High calcium concentrations (0.9 and 1.8 mM) up-regulated the intracellular content of VEGF but down-regulated its release.These results showed a modulated expression and release of VEGF with the stage of keratinocyte differentiation and activation.

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A CD1a POSITIVE XENOGRAFT MODEL FOR RADIOIMMUNOSCINTIGRAPHY.

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Successful imaging of Langerhans Cell Histiocytosis (LCH) in our Phase I trial has prompted the development of a single-chain antibody (scFv) from NA1/34 for both imaging and possible therapy of LCH. Investigations of the effectiveness of an scFv require both *in vitro* and *in vivo* data prior to clinical trials. Thus we have developed a syngeneic mouse xenograft model for the *in vivo* investigation of NA1/34 in order to obtain relevant pharmacokinetic data. This model has been successfully used to study tumour uptake following administration of the agent. CD1a-expressing cell lines used included two stably transfected HeLa lines, $YC1_H$ and $YC1_L$ (both transfected with plasmid PHX15 encoding CD1a under the control of Mo MuLV 5' LTR). HeLa cells were used as a control.

Xenografts were produced by subcutaneous inoculation of female nude (*nu/nu*) mice with $5x10^6$ - $7.5x10^6$ cells in 200µl of RPMI. Animals were monitored daily until tumours measured 6-8mm in diameter. Mice were sacrificed by cervical dislocation, tumours and normal organs were removed. CD1a expression by xenografts was analysed using FITC-labelled NA1/34.

CD1a was detected in $YC1_L$ and $YC1_H$ with expression levels at 5% and 20% of the cell population respectively. Biodistribution of the whole IgG labelled with both ^{111}In and ^{131}I showed specificity towards CD1a positive tumours when compared to CD1a negative xenografts. Tumour specific uptake was 10% injected dose per gram (id/g) for ^{111}In and 7% id/g for ^{131}I labelled antibody.

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PHASE I - RADIOIMMUNOSCINTIGRAPHY OF LANGERHANS CELL HISTIOCYTOSIS.

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Langerhans Cell Histiocytosis (LCH) is a rare disease affecting children and adults, caused by infiltration of cells with the phenotype of epidermal Langerhans cells. The most important marker for Langerhans cells, and the definitive diagnostic marker for LCH is CD1a. Thus CD1a represents a suitable target for immunoscintigraphy of LCH. The purpose of this study is to evaluate the use of the anti-CD1a murine monoclonal antibody NA1/34 labelled with ^{111}In in the localisation of disease in patients with LCH, especially disease activity at sites not amenable to surgical biopsy.

Six adults have been imaged with 1mCi/mg ^{111}In labelled NA1/34. Scans were taken at 2, 24, 48, 72 and 120 hours post injection. One patient displayed specific uptake in cervical lymph nodes, biopsy proven as LCH. A second patient displayed uptake in the 2nd metatarsal, whilst a bone scan also highlighted a previous fracture of the 5th metatarsal. Two further patients with biopsy proven LCH displayed uptake in multiple lymph nodes, previously undiagnosed sites of involvement. Of the remaining patients, one proved to be free from disease while the others images complemented known sites of disease activity.

Our results from this Phase I trial underline the effectiveness of using radiolabelled NA1/34 to highlight areas of active disease. It has allowed the identification of previously undiagnosed sites thus proving a valuable tool in the management of LCH.

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KAPOSI'S SARCOMA-LIKE CELLS FROM PERIPHERAL BLOOD OF KAPOSI SARCOMA PATIENTS CO-EXPRESS CD45 AND MACROPHAGE AND ENDOTHELIAL ANTIGENS. C. Masini, I. Lesnoni La Parola, M. Capuano, A. Diociaiuti, S.Uccini*, L. P. Ruco*, M.C. Sirianni°, L.Vicenzi°, D.Cerimele. Dep. Dermatology UCSC, *Dep. Experimental Medicine and Pathology and °Dep. Clinical Medicine University "La Sapienza", Rome, Italy.

The objective of our study is to investigate the possibility that peripheral blood of Kaposi's Sarcoma (KS) patients contains circulating KS precursor cells.

Peripheral blood mononuclear cells (PBMC) from 5 AIDS-KS and 11 classic KS patients, from 30 healthy volunteers, 5 HIV+ patients without KS, and from 15 patients affected by chronic dermatitis were cultured in 20% conditioned medium from PHA-stimulated PBL for 7-21 days. In positive cultures, adherent spindle cells were detached by trypsinization and were cytocentrifuged. Cytosmears were single and double immunostained with a panel of antibodies. The antigenic profile of PB-derived KS-like spindle cells was compared with that of KS-spindle cells present in 19 tissue biopsies of KS lesions.

Adherent spindle cells were obtained in PBMC coltures of 13/16 KS patients and in none of the controls. KS-like adherent cells co-expressed the endothelial antigens VE-cadherin/CD31 and the macrophage associated antigens Mannose Receptor/CD68/CD14. Their immunophenotype was similar to that detected in the spindle cells of KS skin lesions. Moreover, we have demonstrated by double staining that PB-derived KS-like cells were CD45+/VE-cadherin+. Further evidence suggesting that KS spindle cells are related to the macrophage lineage were provided by the observation that >95% of KS cells present in 19/19 KS tissue biopsies were intensely stained for Mannose Receptor, which is a 175 kDa C-type lectin receptor, selectively expressed by macrophage/dendritic cells.

Our findings are consistent with the possibility that KS lesions derive from local accumulation and proliferation of bone marrow-derived circulating precursor cells, probably belonging to a particular subset of macrophages.

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MEMBRANE-TYPE MATRIX METALLOPROTEINASES-1 (MT-MMP-1): ITS ROLE IN CONNECTIVE TISSUE DEGRADATION. Cornelia Mauch, Dagmar Röckel, Thomas Krieg and Roswitha Nischt, Department of Dermatology, University of Koeln, Koeln, Germany

The newly described membrane-type matrix metallproteinase MT-MMP-1 has been shown to function as a membrane activator of the latent gelatinase A (MMP-2). This protease is belived to play a role during invasion and metastasis of milignant tumors and was shown to be expressed on the surface of lung adenocarcinoma cells.

Fibroblasts and highly invasive melanoma cells which are grown on plastic petri dishes secrete MMP-2 mainly as a proenzyme. Both cell types gain the capability to activate latent gelatinase A to its active 62/59 kDa forms when cells are grown in contact to native collagen type 1 lattices. In order to obtain information whether activation of progelatinase A correlates with the expression of MT-MMP-1, we analyzed specific mRNA levels in human skin fibroblasts and melanoma cells in both cuture conditions. All cells constitutively expressed MT-MMP-1 in various amounts when grown as monolayers. In contrast to fibroblasts showing no change of message levels by culture in collagen lattices, the extracellular matrix induced specific mRNA about 10fold in invasive melanoma cells. This indicates that MT-MMP-1 could contribute to the invasiveness of melanoma cells by activating latent progelatinase A.

In order to address the question whether MT-MMP-1 beside being an activator of MMP-2 can directly degrade matrix proteins, e.g. basement membrane components, we isolated a full length cDNA clone by the RT-PCR technique. With the MT-MMP-1 cDNA driven by the CMV promoter we are currently establishing stable transfected cell lines which will be used for raising antibodies against the recombinant produced protein and for investigating the substrate specificity of MT-MMP-1.

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MICROSATELLITE INSTABILITY- RELEVANCE IN THE PATHOGENESIS OF MELANOCYTIC LESIONS, SPORADIC KERATOAKANTHOMAS AND HIV-ASSOCIATED KAPOSI'S SARCOMAS.

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Microsatellites are widely distributed repetitive DNA sequences and are characterized by heterozygosity. Microsatellites are used to detect loss of heterozygosity (LOH) and to screen for mismatch-repair-defects in hereditary non-polyposis colon cancer (HNPCC) where microsatellite instability (MIN) is common. MIN has been found in sporadic tumors and in keratoakanthomas (KA) in patients with Muir-Torre-syndrome and in patients with HIV-associated Kaposi's sarcomas. Therefore, we evaluated the presence of MIN and LOH in 8 melanocytic nevi, 1 dysplastic nevus, 24 malignant melanomas, 7 metastatic melanomas, 12 KA and 3 HIV-associated Kaposi's sarcomas. After microdissection, isolated DNA from paraffin-embedded material (tumor vs. normal tissue) was examined at 5 separate loci (chromosome 5q (APC), chr.10 (D10S89), chr.11 (D11S904), chr.17p (p53), chr.9p21 (D9S171)). PCR amplification and PAA-gel electrophoresis was performed by standard methods. PCR fragments were visualized with silver staining. MIN was found in 0/8 melanocytic nevi, 0/1 dysplastic nevus, 4/24 malignant melanomas (tumor thickness: 2/13<0,75mm, 2/11>0,75mm), 2/7 metastatic melanomas, 1/12 KA, and 1/3 Kaposi's sarcomas. LOH showed only 2/24 malignant melanomas and 2/7 metastatic melanomas. LOH was found preferable on chromosome 10q (2/24), whereas chromosome 9p21 was not involved. Our results suggest that MIN is an early event in some malignant melanomas, LOH on chromosome 9p21 seems not to be an early event in malignant melanoma and MIN appears not to play an important role in the pathogenesis of sporadic KA.

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TELOMERASE ACTIVITY IN SKIN CANCER. Kazunori Ohnishi, Youichi Takeuchi and Yoshiki Miyachi. Department of Dermatology, Gunma University School of Medicine, Maebashi, Japan.

The specific association of human telomerase activity with immortal cells and cancer has been reported. We investigated telomerase activity in skin cancer and skin cancer *in situ* since the association of the activity with skin cancer has not been well studied. Skin samples of 88 tumors, 5 psoriatic lesions and 17 normal skin samples were studied using TRAP (telomeric repeat amplification protocol) assay. The telomerase activity was detected in none of 5 psoriatic lesions (0%), 8 of 9 squamous cell carcinomas (89%), all of 12 basal cell epitheliomas (100%), 5 of 9 malignant melanomas (56%), 7 of 9 Bowen's diseases (78%), 12 of 14 actinic keratoses (86%) and 4 of 5 extramammary Paget's diseases (80%). On the other hand, 2 of 16 normal skin (13%) and 5 of 11 seborrheic keratoses (45%) also showed detectable telomerase activity, and 6 (86%) of these telomerase positive samples were obtained from sun exposed areas. Ten of 13 melanocytic nevi (77%) and 5 of 6 neurofibromas (83%) also showed telomerase activity. These data indicate that, though telomerase is present in skin cancer and skin cancer *in situ*, the association of the activity with skin malignancy is not specific.

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ANTITUMOR ACTION OF INTERLEUKIN-12, RETINOIDS AND VITAMIN D3 AS RELATED TO THEIR ANTIANGIOGENIC ACTIVITY.

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Tumor cell-induced angiogenesis (TIA) is prerequisite for tumor growth, invasiveness and metastasis, and antiangiogenic factors may be useful in the treatment of angiogenesis-*dependent malignant proliferations.We assessed* antiangiogenic capability of selected compounds known to exert various antitumor effects: interleukin-12, retinoids (all-trans RA, 13-cis RA) and 1,25-dihydroxyvitamin D3 (VD3). TIA was induced in X-ray immunosuppressed Balb/c mice by i.d.injection of human cells: HeLa (from cervical adenocarcinoma), Skv (from bowenoid papulosis), T47D (from breast cancer). Systemic treatment of the mice with retinoids (2.5-5.0mg/kg), VD3 (0.5-1.0 mcg/kg) or IL-12 (0.3-3.0 mcg/mouse) led to significant inhibition of TIA induced by all the cell lines. Combination of IL-12 with retinoids or VD3 synergistically (p< 0.001) decreased TIA, compared to the effects of these compounds used alone. In contrast to previous studies, in our experimental system IL-12 did not decrease body weight of the mice and its antiangiogenic effect could be abolished by i.p.treatment of the mice with antibodies against murine interferon gamma (mIFNg). Further studies will focus on establishing optimal combinations of IL-12, retinoids and VD3 and cytokines for their potential use in the treatment of angiogenesis-dependent disorders.

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ANTITUMOR EFFECT OF PSYCHOTROPIC DRUGS AND RELATED COMPOUNDS

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Some phenothiazines have antitumor activity on Hep-2 cells in vitro. Nitrosourea is a well known anti agent. In this study, we tested the antitumor effects of three series such as 10-nonsubstituted phenothi 10-[n-(phtalimido)alkyl]-1-susbtituted-10H-phenothiazines and 1-(2-chloroethyl)-3-(2-susbtitute phenothazine-10-yl)alkyl-1-ureas with H, Cl and trifluoromethyl (CF_3) substitution on the second carbon of the phenothiazine ring. Structural combination with urea, connected with propyl-, or butyl bridge tricyclic ring and modification with H, Cl or CF_3 were accomplished in a hope that these derivatives wi stronger antitumor effect. We tested the antiproliferative effect of these derivatives on human epithelial cells of the larynx (Hep-2) *in vitro*. Greiner 96-well flat bottom microtrays were used, each well con 5000-8000 cells. Concentrations from 1mg/ml to $3.125x10^{-2}$ mg/ml dilutions of the drugs were added t well (0.1ml/well). After 4 days of incubation at 37 °C in a CO_2 incubator cells were fixed and staine 0.25% crystal violet. The Inhibition Dose 50 (ID_{50}) was determined by measuring the optical density nm. Among the phenothiazines substituted with H, Cl or CF_3 at position 2, the CF_3 derivative showed th antitumor effect (ID_{50}=4.7μg). Among the other group combined with urea, the Cl and CF_3 subs derivatives inhibited the malignant cells best.There was not a *big difference whether it was a* (ID_{50}=6.3μg) or butyl (ID_{50}=7.8μg) bridge between the urea and the tricyclic skeleton when it was susb with chlorine, but there was a difference in the cell inhibition due to the length of alkyl bridge wh substitution was CF_3. The derivative with butyl bridge (ID_{50}=7.3μg) was 2-fold more effective inhibiti *growth than with propyl bridge* (ID_{50}= 18.8μg). *In summary*, substitution with CF_3 was always the h inhibiting malingnant cell growth, but there was difference if the phenothiazine ring was connected to th with propyl-, or butyl bridge. This could be in connection with the intercalation into the DNA. Subst with Cl and combination with urea was almost 10 fold more effective than without urea. This could refer additive interaction between phenothiazine and urea. These newly synthetized drugs with pro antiproliferative effect need further tests.

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CLONALLY EXPANDED T CELL RECEPTOR MOTIFS OF PSORIATIC SKIN LESIONS ALSO RESIDE IN CLINICALLY HEALTHY SKIN

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Psoriasis vulgaris is now considered as a disease of abnormal keratinocyte proliferation induced by T lymphocytes. T cells dominate the dense mononuclear infiltrate of psoriatic skin lesions (PSL), but are also observed in a somewhat greater abundance in clinically normal skin (NPS) of patients with active psoriasis than in skin of healthy individuals (HS). We demonstrated in a recent study (Menssen et al., J. Immunol. 155:4078-4083, 1995) that T cells of psoriatic infiltrates are characterized by a marked oligoclonal T cell receptor (TCR) repertoire representative of antigen-driven T cell expansion, while corresponding blood samples or HS showed no signs of clonal selection. To determine the nature of the skin homing T cells in NPS we compared the TCR repertoire in PSL and NPS by semiquantitative TCR-Vß-specific PCR and sequence analysis of the dominant Vß families in two individuals suffering from sever chronic plaque type psoriasis. Clonally expanded TCR motifs were observed in overexpressed Vß6 TCR families of PSL. The same TCR sequences were also detected in NPS but at lower frequency. Thus, clonally expanded T lymphocytes of PSL also reside in NPS were the may become activated by the putative psoriatic antigens to expand and initiate new psoriatic lesions.

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SKIN-HOMING T LYMPHOCYTES PREFERENTIALLY REARRANGE Vß2 OR Vß6 T CELL RECEPTORS WITHOUT EVIDENCE FOR DOMINANT CLONAL SELECTION

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T lymphocytes in epithelial organs comprise an essential part of the immune system. They are positioned to serve as first line of defense against external pathogens. To investigate whether skin-homing T cells (SHTC) are subject to antigenic selection forces distinct from that shaping the T cells in peripheral blood (BTC) we performed a comparative analysis of Vß gene segment usage in normal skin and paired blood samples by semiquantitative PCR. Primers for 20 different human TCR Vß gene families were used to analyze the repertoire of TCR ß chain transcripts in SHTC and corresponding BTC of six individuals. In all skin samples the dominating Vß gene families were either or both Vß2 and Vß6, while BTC showed a more even Vß distribution. Sequence analysis of the predominant Vß2 and Vß6 families in all six individuals revealed no evidence for a dominant antigenic T cell stimulus but exhibited a broad polyclonal T cell receptor repertoire. Thus, as compared to the corresponding BTC, SHTC preferentially rearrange Vß2 or Vß6. Unlike the intestinal T cell population the repertoire of SHTC is not shaped by a limited set of foreign or self antigens but rather by polyclonal stimulators such as superantigens.

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ANALYSIS OF T CELL RECEPTOR EXPRESSION IN EARLY CHRONIC PLAQUE PSORIASIS BY A REPRODUCIBLE ANCHORED POLYMERASE CHAIN REACTION AND DOT BLOTTING METHOD.

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We have developed an efficient and reliable method for the analysis of T cell receptor β (TCRβ) chain expression in peripheral blood mononuclear cells and 4mm lesional punch biopsies from psoriasis patients. mRNA extracted from the blood and biopsies is expanded by a two-stage semi-nested anchored polymerase chain reaction (PCR) method and immobilised onto nitrocellulose membrane by dot-blotting. Hybridisation with a panel of 25 Vβ family specific oligonucleotide probes then allows the generation of a Vβ profile for each sample. By rigorously controlling all steps of the assay we have been able to achieve reproducibility of between 0.2 and 2.8% in duplicate assays of the same mRNA. Any apparent expansion of a Vβ family in the skin as compared with the peripheral blood is then further investigated for evidence of clonal T cell expansion by CDR3 size spectratyping of cDNA expanded with appropriate Vβ family-specific primers, on denaturing acrylamide gels.

Analysis of the peripheral blood and early psoriatic lesions (less than three weeks old) of 10 chronic plaque patients showed a significantly increased percentage of Vβ2 (two patients), Vβ6 (two patients), Vβ10 (one patient) and Vβ14 (one patient) in the skin as compared with the peripheral blood (each Vβ forming at least 10% of the total skin population). We did not find evidence of the consistent and large increase in Vβ2 which has been reported by others. CDR3 spectratyping of the increased Vβ families in these patients did not show any evidence of significant clonal T cell expansion. This data indicates that there is no consistently increased expression of a single Vβ species or significant clonal expansion in early psoriasis lesions when unfractionated T cell populations are analysed.

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LESIONAL PSORIATIC T CELLS CONTAIN THE CAPACITY TO INDUCE A T CELL ACTIVATION MOLECULE CDw60 ON NORMAL KERATINOCYTES.

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CDw60 is expressed on about 75% T cells in psoriatic skin but only on approximately 25% periferal blood T cells. In psoriatic skin basal and suprabasal keratinocytes also express CDw60. The CDw60 mAb UM4D4, has been shown to recognize the 9-O-acetylated disialosyl group on ganglioside GD_3. The binding of anti-UM4D4 was markedly reduced after neuraminidase treatment of keratinocytes, indicating that CDw60 on cultured keratinocytes also is a part of the 9-O-acetylated disialosyl group. To examine whether factors from T cells in psoriatic lesions are responsible for the enhanced expression of CDw60, lesional T cell were cloned. Factors released from 19 out of 19 activated T cell clones upregulated CDw60 expression on cultured keratinocytes (from 22±12% to 42±18%, mean±SD, p<0.01). T-cell secreted cytokines, including IL-2, IL-3, IL-4, IL-13, TGF-β, GM-CSF and IFN-γ were tested for their capacity to modulate keratinocyte CDw60 expression. IL-4 and IL-13 strongly upregulated the expression of CDw60 (IL-4: from 14±10% to 48±13%, n=8, p<0.05; IL-13: from 46±13% to 76±16%, n=9, p<0.01); by contrast, IFN-γ downregulated keratinocyte CDw60 expression (from 32±21% to 16±20%, n=6, p<0.05). IL-13 may be partially responsible for the T cell induced upregulation of CDw60, because anti-IL-13 partly neutralized this effect of the T cell supernatant. Anti-UM4D4 is mitogenic for preactivated T cells, so we next determined whether anti-UM4D4 also induced proliferation of CDw60⁺ keratinocytes. Although anti-UM4D4 did not induce proliferation of normal keratinocytes, anti-UM4D4 did induce proliferation of a CDw60⁺ squamous carcinoma cell line. In conclusion, CDw60 expression on psoriatic keratinocytes is likely induced by intralesionally-activated T cells and may be involved in keratinocyte hyperproliferation in psoriasis.

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CD40 IS FUNCTIONALLY EXPRESSED ON HUMAN KERATINOCYTES (KC).

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The CD40/gp39 pathway is known to be an important feature of B - T cell collaboration leading to T cell-dependent activation, proliferation or differentiation of B cells. Additionally, CD40 is involved in the regulation of B cell survival and apoptosis. Recently, CD40 has been shown to be expressed functionally on non-hematopoetic cells, i. e. endothelial cells. Here, we demonstrate that human keratinocytes, cultured in vitro, express CD40 constitutively. This surface expression of CD40 is markedly upregulated following stimulation with IFN-γ, but not with TNF-α or IL-1β. This process is regulated at the CD40 mRNA level as demonstrated by Northern blot analysis. Furthermore, ligation of CD40 via soluble gp39, the CD40 ligand, enhances ICAM-1 and Bcl-x upregulation on IFN-γ stimulated KC, but not LFA-3, B7-2, HLADR, or Fas expression. The release of cytokines is also induced following CD40 ligation. In psoriasis, a T cell-mediated inflammatory skin disease, KC have markedly enhanced their expression of CD40. This expression co-localizes with the expression of ICAM-1, Bcl-x, and an influx of $CD3^+$ T cells. These findings suggest a functional role of CD40 on KC in inflammatory skin disorders, i. e. psoriasis, and therefore intervention with the CD40/gp39 pathway could be a new therapeutic approach in inflammatory skin disorders like psoriasis.

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ANTHRALIN ACTIVATES THE TRANSCRIPTION FACTOR NF-κB MEDIATED BY REACTIVE OXYGEN SPECIES.

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Anthralin, 1,8-dihydroxy-9-anthrone, is an effective and widely used therapeutic agent for the topical treatment of psoriasis. Anthralin is known to induce an inflammatory response in the skin which is thought to be essential for its therapeutic efficiency. NF-κB is a transcription factor that activates an array of inflammatory genes in response to a variety of stimuli. In this study we show that as little as 10 μM anthralin activate NF-κB. This activation was inhibited by N-acetyl-cysteine and pyrrolidinedithiocarbamate. Furthermore, in keratinocytes stably overexpressing catalase, a hydrogen peroxide degrading enzyme, a significant reduction of NF-κB activation was found, while stable overexpression of Cu/Zn superoxide dismutase, a cytosolic enzyme which degrades superoxide generating hydrogen peroxide, augmented the anthralin effect. We conclude from these results that anthralin induces NF-κB via reactive oxygen species and in particular that hydrogen peroxide seems to be the second messenger. The skin reactions that are known to occur after anthralin treatment can be explained as result of the transcriptional induction of cytokines and adhesion molecules regulated by NF-κB. An explanation for the antiproliferative effect on psoriatic plaques could be the induction of apoptosis by NF-κB. Bone marrow cells infected with c-Rel expression vectors have been shown to undergo apoptosis. We could show that the anthralin induced NF-κB complex consisted of p50, p65 and c-Rel. In summary, we suggest that the reactive oxygen species induced activation of NF-κB plays a significant role in the anti-psoriatic activity of the drug anthralin.

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CERAMIDES OF THE PSORIATIC SCALE: FATTY ACIDS COMPOSITION

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Quali and quantitative analysis of ceramide bands in psoriatic scale compared to normal human stratum corneum was the object of previous works. The aim of this study is the correlation between thin layer chromatographic (TLC) behaviour of ceramide bands and their structural characteristics. For this purpose 8 ceramide spots from psoriatic scale and 7 from normal human stratum corneum were isolated (1) and fatty acid composition of single ceramide fractions analysed by capillary gas liquid chromatography (GLC). After methanolysis, the samples were heated according to the following GLC temperature program: 4 min at 150°C; a temperature increase of 4°C/min in order to reach 250 °C and then standby for 31 min. The capillary column used is a DB5 (J&W-Fisons) installed in a GC8000 Fisons gas chromatograph. We investigated free fatty acids from C16:0 to C28:0 and α-hydroxy free fatty acids from C16:0 to C26:0, identified according to Sigma standards. The data obtained are expressed as weight percent. The results of normal human stratum corneum ceramides are in good agreement with published works, apart from the most polar ceramides (CER V, CER $VI_{1,2}$) and the inusual esterified ceramide I. The results concerning psoriatic scale ceramides are intriguing since CER $V_{1,2}$ and CER $VI_{1,2}$ appear not to be coincident with their TLC chromatographic behaviour. In fact the ratio whole chain length/whole chain hydroxylation in CER $V_{1,2}$ is 3/7 while in CER $VI_{1,2}$ is viceversa. Our hypothesis is that whole ceramide chain length plays a more important role in TLC mobility than ceramide hydroxylation, commonly considered the main factor of polarity. Thus, in order to have a precise interpretation of ceramides, the following parameters were evaluated : chain length; hydroxilation; chromatographic mobility; weight percent.

In conclusion ceramide physiological role and their pathological implication will be better explained considering these compounds in an holistic way.

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DEMONSTRATION OF HEPOXILINS AND TRIOXILINS IN THE SCALE EXTRACTS OF PSORIASIS.

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Hepoxilins and trioxilins are eicosanoids formed through 12-lipoxygenase pathway. We recently found that normal human epidermis produces high amounts of hepoxilin B_3, and trioxilins A_3 and B_3 **in vitro** [J Mass Spectrom and Rap Commun Mass Spectrom. 1995; S169-S182]. The aim of the present work was to evaluate the presence of 12-lipoxygenase derived eicosanoids other than 12-hydroxy-eicosatetraenoic acid (12-HETE) in psoriatic scales. Scales from patients with chronic stable plaque psoriasis involving more than 10% of body surface, were extracted with organic solvents and analysed by a combination of HPLC and GC-MS techniques. In normal epidermis, trioxilins were not detected and only small amount of hepoxilin B_3 was found (0.12±0.06 ng/mg epidermis). In psoriatic scales we found 17-fold more hepoxilin B_3 than in normal epidermis (ratio of hepoxilin B_3 to 12-HETE: 0.19±0.09) and also trioxilins A_3 and B_3 (ratios of trioxilin A_3 and B_3 to 12-HETE: 0.65±0.23 and 0.315±0.28 respectively). Our results demonstrate that other 12-lipoxygenase derived eicosanoids are produced **in vivo** by human skin and that these compounds are increased in psoriasis. The biological role of hepoxilins is not fully understood, but reported data suggest that hepoxilins play a role as intracellular and intercellular signalling molecules. Our results suggest that these compounds could play a role as mediators in the inflammatory response on skin, particularly on psoriasis.

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EPICUTANEOUS APPLICATION OF LEUKOTRIENE B4 INDUCES PATTERNS OF TENASCIN AND A HEPARANSULFATE PROTEOGLYCAN EPITOPE THAT ARE TYPICAL FOR PSORIATIC LESIONS.

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Application of leukotriene B4 (LTB4) to normal human skin induces changes similar to those found in psoriatic skin, and it proved to be a useful model to study the pathogenesis and treatment of psoriasis. Here we have studied the expression patterns of molecules that were recently shown to be altered in lesional psoriatic skin, including the extracellular matrix protein tenascin (TN) and the basement membrane and cell-surface associated heparan sulfate proteoglycans (HSPGs).

During a 72h time course the expression of these markers was studied immunohistochemically and was correlated with epidermal proliferation and influx of inflammatory cells. Following the peak influx of polymorphonuclear leukocytes, a marked increase in TN expression was noted in the papillary dermis at 72 h after LTB4 application. The expression patterns of basal membrane associated epitopes of HSPG remained unaltered, whereas the expression of cell surface associated HSPG disappeared at 72 hours after LTB4 application. A significant correlation was found between dermal TN expression and epidermal hyperproliferation, and between TN expression and the presence of dermal T-cells.

These findings indicate that the model of LTB4 induced acute cutaneous inflammation displays many characteristics of the early psoriatic lesion and could serve as a model to study the cell biological changes in this disease.

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TRANSGLUTAMINASE EXPRESSION IN LESIONAL SKIN, DISTANT CLINICALLY UNINVOLVED SKIN, AND IN THE MARGIN ZONE OF PSORIASIS.

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Plasma membrane bound transglutaminase is a key enzyme in the formation of the cornified envelope. In lesional skin of psoriatic patients it has been demonstrated that the activity of this enzyme is increased. The aim of the present study was to investigate the topographical distribution of plasma membrane bound transglutaminase in lesional skin, distant clinically uninvolved skin and the margin zone of spreading lesions in patients with psoriasis.

In lesional epidermis the number of transglutaminase positive cell layers is markedly increased compared to the distant clinically uninvolved skin. In patients with stable psoriasis the number of transglutaminase positive cell layers was more increased compared to patients with spreading psoriatic plaques. In the transition zone between symptomless and lesional skin, the number of transglutaminase positive cell layers increased gradually in contrast to the sharp on/off transition with respect to parakeratosis, involucrin, filaggrin and recruitment of cycling epidermal cells, as we described in an earlier investigation. Transglutaminase proved to increase further proximally to the transition point of the latter parameters. It is feasible that the expression of transglutaminase is an adaptation to psoriatic dysregulation and not a cause.

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LACK OF ASSOCIATION OF SKIN-DERIVED ANTILEUKOPROTEINASE/ELAFIN GENE POLYMORPHISM WITH DIFFERENT FORMS OF PSORIASIS.

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Skin-derived antileukoproteinase (SKALP), also known as elafin, is a potent inhibitor of leukocyte elastase and proteinase 3, two neutral proteinases that have been implicated in tissue destruction and leukocyte migration. SKALP is absent in normal epidermis but is induced under inflammatory conditions. We have recently shown that, at least at the protein level, SKALP is significantly decreased in lesional skin of pustular psoriasis compared to plaque type psoriasis. This finding raised the possibility that SKALP could be one of the candidate genes for pustular forms of psoriasis. We therefore performed single strand conformation polymorphism analysis to screen for mutations/polymorphisms in the exons of 30 patients with plaque type psoriasis, 15 patients with pustular psoriasis and 48 healthy controls. No mutations/polymorphisms were detected in any of these groups. However, in the promoter region of the SKALP gene, a dinucleotide repeat polymorphism was identified with a frequency of 27% heterozygotes in the control population. In both groups of psoriatic patients a similar frequency was found. We conclude that the decrease in SKALP activity in pustular psoriasis is not caused by mutations in the coding region of the gene, and that there is no allelic association between psoriasis and a polymorphism found in the promoter region.

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***IN VITRO* INDUCTION OF NORMAL AND PSORIATIC PHENOTYPES**

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Lesional psoriatic epidermis displays a number of phenotypic changes that are distinct from the differentiation program found in normal interfollicular epidermis. In psoriatic epidermis, keratinocytes are hyperproliferative and several differentiation-associated molecules are expressed that are absent in normal skin (e.g. cytokeratins (CK) 6, 16 and 17, and the epidermal proteinase inhibitor SKALP/elafin). In addition, several molecules which are normally restricted to the stratum granulosum are strongly upregulated in the stratum spinosum (e.g. psoriasis-associated fatty acid binding protein (PA-FABP), psoriasin, involucrin and transglutaminase). The aim of this study was to develop *in vitro* culture systems which (a) would allow to study the induction of normal and psoriatic differentiation pathways and (b) would be amenable for screening of antipsoriatic drugs. Here we have investigated several models for induction of differentiation with respect to the expression of markers for the normal and psoriatic phenotype. Cell cycle parameters and expression levels of CK1, CK10, CK16, SKALP/elafin, transglutaminase, involucrin, psoriasin, and PA-FABP were assessed in these models using flow cytometry, immunocytochemistry and northern blot analysis. We observed that induction of differentiation with fetal calf serum resembled the psoriatic phenotype (sustained hyperproliferation; high levels of CK16, SKALP/elafin, transglutaminase and involucrin; moderate psoriasin expression), whereas differentiation induced by growth factor depletion in a confluent culture resembled the normal differentiation phenotype (low proliferative rate; high expression levels of CK1 and 10; moderate expression of involucrin and transglutaminase; low expression levels of SKALP/elafin and CK16; absence of psoriasin). We propose that these models can be used to study expression and pharmacological modulation of selected differentiation genes and the coordinated expression of sets of genes associated with epidermal differentiation programs.

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DISTRIBUTION OF THE CD11B/CD18 INTEGRIN AND ITS LIGAND ICAM-1 IN THE MARGIN OF THE SPREADING PSORIATIC PLAQUE.

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In polymorphonuclear leukocytes (PMN) the β2-integrin-receptormolecule Mac-1 (CD11b/CD18) is of particular importance for performing an adequate inflammatory response. To obtain more insight in the contribution of CD11b, CD18 and ICAM-1 in the kinetics of migration of inflammatory cells in instable psoriasis, we studied their distribution in the margin of the actively spreading psoriatic lesion. In eight untreated patients punchbiopsies were taken from the margin which was defined as the transition point from ortho- into para-keratotic stratum corneum. Additional biopsies were taken from central psoriatic and distant uninvolved skin, as well as normal skin from healthy volunteers. The localisation of PMN was assessed using anti-elastase and all markers were quantified using immunohistochemical methods. Involved psoriatic skin showed a significant increase compared to uninvolved skin of nearly all parameters. CD18 was most abundantly present. All dermal changes preceded those in the epidermis: regarding the margin zone from the uninvolved side, the first change was a decrease in ICAM-1-expression. This could imply masking or presence of a receptor more important in the early inflammatory reaction. More centrally an increase in CD11b-expression was observed. CD11b showed a different tissue distribution from elastase and outnumbers elastase in distant uninvolved skin, suggesting significant amounts of other CD11b-bearing cell populations than PMN. Additionally, elastase could have been digested by skin derived anti-leukoproteinase (SKALP), known to be present in psoriatic skin. Most centrally an increase in CD18-expression occurred. The CD18-expression of the distant uninvolved skin was decreased compared to normal skin which is compatible with an abnormal trafficking of leukocytes within the dermis. In the central part of the psoriatic lesion the ratio of CD11b and CD18 is constant suggesting a relevant contribution of Mac-1 in the dermal migration of inflammatory cells.

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IN PSORIATIC SKIN THE BINDING TO A RESPONSE ELEMENT CORRELATE WITH THE LEVELS OF THE VITAMIN D RECEPTOR AND THE RETINOID X RECEPTOR. Tina J. Jensen, Sif Sørensen, Henrik Sølvsten, Knud Kragballe,
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The genomic effects of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are mediated through the vitamin D receptor (VDR), which binds to response elements (RE) in the promotor region of responsive genes. In most genes the VDR interacts with the RE by forming a heterodimer with the retinoid X receptor (RXR). The purpose of this study was to determine the levels of VDR and RXRα and their binding to a RE consisting of 2 direct repeat hexanucleotides spaced by 3 nucleotides (DR-3) in psoriatic skin. Matched keratome biopsies were obtained from uninvolved (UN) and involved (IN) skin from patients (n=8) with plaque-stage disease. Protein extracts were subjected to Western analysis using the monoclonal antibodies 9A7γ and 4RX-1F6 against VDR and RXRα, respectively. The levels of binding to the DR-3 RE were determined by the electrophoretic mobility shift assay (EMSA). The mean levels of VDR and RXRα as well as the binding to the DR-3 RE were similar in UI and IN. There was a strong correlation between the VDR levels and the binding to the DR-3 RE ($r=0.77$; $p=0.026$) and between the RXRα levels and the binding to the DR-3 RE ($r=0.83$; $p=0.005$). These results indicate that the genomic effects of $1,25(OH)_2D_3$ in the skin are not only dependent on the VDR levels, but also on the levels of the heterodimeric partner RXRα. Fluctuations in RXRα levels may, therefore, modulate the responsiveness to $1,25(OH)_2D_3$ in psoriatic skin.

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ELEVATED CIRCULATING VASCULAR ENDOTHELIAL GROWTH FACTOR IN ERYTHRODERMA. D Creamer, MH Allen, RW Groves*, JNWN Barker St John's Institute of Dermatology, UMDS and *Dept of Dermatology, University College, London, UK.

Angiogenesis and vascular hyperpermeability are prominant features in psoriasis while increased capillary leakage is observed in other inflammatory dermatoses. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine possessing potent angiogenic activity as well as acting directly on microvasculature to increase permeability. Epidermal overexpression of VEGF has been demonstrated in plaque psoriasis, however no data exists on the presence of circulating VEGF in cutaneous diseases.

Plasma samples from patients with erythrodermic eczema (E-ec, n=5), erythrodermic psoriasis (E-ps, n=9) and normal individuals (n=17) were immunoassayed for human VEGF using a commercially available quantitative sandwich ELISA kit. Plasma levels of VEGF were significantly elevated in erythrodermic patients compared to controls (E-ps/E-ec: mean=31.9 +/-6.6 SEM, normal: mean=8.3 +/- 2.5 SEM. $P<0.005$, two-sample t-test), and in erythrodermic psoriatics compared to normals (E-ps: mean=28.5 +/- 7.8 SEM, normal: mean=8.3 +/- 2.5 SEM. $P<0.05$).

Circulating VEGF in erythroderma is likely to reflect absorption into the dermal microcirculation from extensive cutaneous disease. The measurement of systemic levels of VEGF may represent an important prognostic parameter in erythroderma.

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ENHANCED APOPTOSIS IN PSORIATIC EPIDERMAL CELLS. IL-β maturation is related to apoptosis *ex vivo*.

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Non-stimulated, short-term primary cultures of epidermal cells (EC) (*ex vivo* model) from lesional psoriatic (PP) skin secrete functionally active IL-1β in contrast to EC from normal controls (NN). The generation of functionally active, mature IL-1β and its secretion depend on IL-1β processing. As IL-1β processing is a feature of apoptosis, at least in monocytic cells, this study was conducted to investigate whether IL-1β maturation in PP EC is related to apoptosis. Analysis of intra- and extracellular IL-1 revealed that the increased secretion of IL-1β by PP EC was due to increased *de novo* synthesis of IL-1β, which was inversely correlated with trypan blue positivity of EC *ex vivo* (= necrosis). In fact, apoptosis was increased in freshly isolated PP EC relative to NN EC, as measured by TUNEL and ELISA specific for cytosolic internucleosome-sized DNA fragments. Double-staining of PP EC for CD45 showed that intra-epidermal leukocytes were not responsible for the observed DNA end-labeling. In our experiments, DNA fragmentation represented genuine apoptosis as kinetic studies with PP EC showed that DNA fragments were most abundantly present intracellularly, showing peak levels at 8 h, which clearly preceded those of extracellular DNA fragments at 24 h after seeding EC. The amounts of cytosolic and extracellular IL-1β, as measured by ELISA, paralleled those of DNA fragments. Moreover, the use of the D10 bioassay combined with neutralizing antibodies revealed a nice correlation between released biologically active IL-1β and DNA fragmentation in PP EC. In accordance to the findings *ex vivo*, the *in situ* DNA end-labeling was enhanced in PP skin when compared to NN skin, and was localized to the upper stratum granulosum. In a few patients IL-1β staining clearly co-localized with DNA end-labeling. Our studies provide evidence that resident PP EC express enhanced apoptosis and suggest that these cells are a source of mature IL-1β synthesized *de novo*.

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TREATMENT OF PSORIASIS VULGARIS WITH CALCIPOTRIOL CREAM AND TOPICAL CORTICOSTEROIDS: A COMPARATIVE FLOW CYTOMETRIC STUDY. Conrad P. Glade, Piet E.J. van Erp, Peter C.M van de Kerkhof, Department of Dermatology, University Hospital Nijmegen, The Netherlands.

A recently developed flow cytometric multiparameter technique with simultaneous assessment of markers for proliferation (TO-PRO-3), differentiation (anti-keratin 10) and inflammation (anti-vimentin) was used to evaluate therapy modalities consisting of calcipotriol and topical corticosteroids. Forty patients with a chronic plaque psoriasis were at random divided in four parallel groups and treated for 8 weeks with 1) calcipotriol cream (50 μg/g once daily), 2) calcipotriol cream twice daily, 3) calcipotriol and clobetasone (0.5 μg/g) creams and 4) calcipotriol and betamethasone (1 mg/g) creams. Before and after treatment keratotome biopsies were taken and single cell suspensions prepared for flow cytometric analysis.

A decrease of the percentage of basal cells in S- and G_2M phase (proliferation) was obtained with all therapy modalities (3.2% to 7.8%). However, this decrease was not significant in the calcipotriol once daily group. A highly significant reduction of vimentin positive cells (non-keratinocytes) was observed following combined treatment with calcipotriol and clobetasone. In contrast, monotherapy with calcipotriol had virtually no effect on the number of vimentin positive cells. We conclude that the addition of topical corticosteroids significantly enhances the antiinflammatory potential of calcipotriol.

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RIGHT/ LEFT COMPARISON OF DITHRANOL MONO THERAPY AND CALCIPOTRIOL/ DITHRANOL COMBINATION THERAPY IN CHRONIC PLAQUE PSORIASIS. Katja Stephanek*,**, Khusru Asadullah* and Wolfram Sterry*, Departement of Dermatology, Charité Berlin* and Schering AG Berlin**, Germany.

Dithranol is an old, widely used, effective treatment for chronic plaque psoriasis. For in-patients it is regarded as first-line topical treatment. The new vitamin D analogue calcipotriol on the other hand- causing no staining- is better accepted for out-patients treatment. Our question was, whether calcipotriol ointment (Psorcutan®) applied once daily is additive to classical dithranol mono therapy. Therefore, 10 in-patients with chronic plaque psoriasis were treated with dithranol (highest concentration tolerated, right and left body side) in the morning and calcipotriol ointment (50μg/g) just on the left body side in the evening for 4 weeks. Efficacy was measured at baseline and on days 4, 8, 11, 15, 22 and 29 by visual scoring and 20 MHz ultrasound. Ultrasonographic examination showed a reduction of the echopoor band (corresponding to the sum of acanthosis and infiltrate in the upper dermis) for both treatments as early as day 4. There was a statistically significant difference between both treatments in favour of the combination therapy at day 8 and remained so at subsequent assessements. Both treatments were well tolerated. Therefore calcipotriol ointment used once daily had an additive effect to classical dithranol mono therapy in a pilot study including 10 patients suffering of chronic plaque psoriasis. This has to be confirmed by further studies including higher numbers of patients .

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An Immunohistological Study On Infant Generalized Pustular Psoriasis Improved With Vitamin D_3 Ointment And Maintenance Treatment With Vitamin D_3 And Colchicine In Combination.

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A female infant with generalized pustular psoriasis was treated with vitamin D_3 ointment alone. The dose of the ointment was gradually decreased. The remission could be mainfained with a low dose of vitamin D_3 ointment and colchicine in combination. The clinical course is hereinafter reported, as well as immunohistological findings.

(Case) A four-year-old female infant. She had pustular psoriasis in October, 1995, followed by generalization of squamores erythema. In histological observation of the skin, parakeratosis associated with munro abscess was found in epidermis, intensive edema containing neutrophils and lymphocytes in dermal papillae and perivascular lymphocytic infiltration in mid-dermis. Granular layer disappeared beneath microabscess. CD_4-positive cells were predominantly found among infiltrating lymphocytes. ELAM-1 and ICAM-1 were detected only in vascular endothelium and VCAM-1 was positive in both intravacular cells of dermis and vascular endothelium. After treatment, CD_4 positive T cells were the most predominant, and the population of CD_4 positive T cells was almost equal to that of CD_8 positive T cells and only vascular endothelium was positive for VCAM-1.

(Summary) For generalized pustular psoriasis in infants, it is and will be difficult to indicate immunodepressive therapy, and, even if possible, it can be indicated only for short term and the disease recurs. The combination of vitamin D_3 dose-reducing therapy with colchicine may assure long-term remission and this combined therapy was also found to be immunohistologically useful.

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EXPRESSION OF ADHESION MOLECULES IN PSORIATIC SKIN: EFFECTS OF TOPICAL APPLICATION OF VITAMIN D ANALOGUES.

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Increasing evidence suggests involvement of adhesion molecules in the pathogenesis of psoriasis, contributing to uncontrolled keratinocyte proliferation, neovascularization, and invasion of inflammatory cells. We have analyzed immunohistochemically the *in situ* expression of VLA-integrins (CD29, CDw49b, CDw49c, CDw49e, CDw49f), E- and P-Cadherins, and CD44 isoforms on frozen sections of normal and psoriatic skin (nonlesional skin, lesional skin before and along with topical treatment with calcitriol or analogues). We did not observe visual changes of immunoreactivity in nonlesional psoriatic as compared to normal skin, while the staining pattern of CDw49b, CDw49c, CDw49f, and CD29 was severely altered in untreated lesional psoriatic skin. Most markedly, these adhesion molecules were focally upregulated in suprapapillar epidermal compartments of lesional skin, a staining pattern that is in accordance with the phenomenon described by Pinkus as "squirting papilla". After eight weeks of treatment with calcitriol or analogues, the staining pattern for these adhesion molecules was markedly changed, aligning to the pattern characteristic for nonlesional psoriatic or normal skin, although expression of CDw49f and CD29 was still upregulated in the dermal compartment. Our findings demonstrate: (i) that expression of adhesion molecules is severely altered in lesional but not in nonlesional psoriatic skin and (ii) that topical treatment with calcitriol or analogues modulates expression of adhesion molecules in psoriatic skin.

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TACALCITOL INHIBITS THE INCREASE OF TRANS-EPIDERMAL WATER LOSS INDUCED BY PHORBOLESTER IN HAIRLESS MICE.

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Tacalcitol (1,24(R)-$(OH)_2D_3$, TV-02) was synthesized by Teijin Institute for Bio-Medical Research, Teijin Limited and has been reported to inhibit *proliferation and induce differentiation of keratinocytes in vitro and in vivo*. The ointment preparation of tacalcitol (2 μg/g) has been shown to be effective in psoriasis and already launched in Japan. Recently, we reported the anti-inflammatory effect of tacalcitol in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated hairless mice. In this study, we examined the effects of tacalcitol on trans epidermal water loss (TEWL) in comparison with steroids and calcipotriol (MC-903) in this model.

Female hairless mice (SKH1 strain, Crl.) was used in this study. The TEWL was measured after the topical application of TPA (10 nmol/mouse) or TPA plus tacalcitol, calcipotriol, or steroid to the dorsal skin of hairless mice.

TPA increased the TEWL from 48 hour after application, and tacalcitol significantly inhibited the TPA-induced TEWL from the dose of 1 ng/mouse to 100 ng/mouse. The ointment preparation of tacalcitol (2 μg/g) also significantly inhibited the TEWL. Tacalcitol was as strong as prednisolone (50 μg/mouse or 5 mg/g ointment) or betamethasone valerate (1.2 mg/g ointment), and stronger than calcipotriol (100 ng/mouse or 50 μg/g ointment).

Our study showed the efficacy of tacalcitol for suppressing TPA-induced TEWL in hairless mice. These findings suggest that tacalcitol has the effects not only on epidermal proliferation, differentiation or inflammation, but also on skin barrier function in psoriasis and other inflammatory skin disorders.

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TOPICAL PHOTODYNAMIC THERAPY OF PSORIASIS USING A NOVEL PORPHYCENE DYE

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Topical Photodynamic Therapy (tPDT) with aminolevulinic-acid (ALA), an endogenous photosensitizer, has proven to be effective in the treatment of non-melanoma skin cancer and inflammatory diseases like psoriasis. 9-Acetoxy-2,7,12,17-tetrakis(ß-methoxyethyl)porphycene (ATMPn) is a new exogenous photosensitizer, that has shown superior in vitro and in vivo characteristics as compared to ALA. To evaluate efficacy and safety of tPDT with ATMPn an early phase II clinical study was conducted including 29 patients with stable plaque-type psoriasis.

Prior to therapy psoriatic plaques were debrided and scored for infiltration, erythema, elevation and scaling. An 0.1% ethanolic ATMPn formulation was applied topically (under occlusion vs. non-occlusion) for 2h or 24h on four separate plaques of each patient. Thereafter irradiation of two plaques was performed using an incoherent light source (12.5-100 J/cm² / 30-90 mW/cm²). Biopsies of the other two plaques were taken to study penetration of the drug into the epidermis.

Best results were achieved following incubation for 24h under occlusion and irradiation with 45-90 J/cm² and 30-90 mW/cm². A significant score reduction was seen in these patients 1-4 weeks after therapy, lateron a recurrence of psoriatic lesions occured. The only side-effects were erythema and edema of the incubated area following irradiation. Fluorescence image analysis of the biopsy sections showed intense red fluorescence indicative for ATMPn in stratum corneum after 2h of incubation and also of upper parts of epidermis after 24h. These results were confirmed quantitatively by digital image analysis.

This report shows the potential efficacy and good tolerability of tPDT with ATMPn for the treatment of psoriasis. In contrast to PUVA, tPDT does not have the potential risk of inducing skin malignancies. However, no complete remission was achieved using a single treatment protocol. Therefore repeated treatments will be used in further studies to achieve a prolonged remission of psoriasis.

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FUMARIC ACID DERIVATIVES DIFFERENTIALLY INFLUENCE THE METABOLIC ACTIVITY AND CHEMOKINE SECRETION OF LONG TERM CULTURED HUMAN PERIPHERAL CD3 + T LYMPHOCYTES.

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Fumaric acid derivatives have been effectively used for the treatment of psoriasis for many years . The mode of action, as well as the main target cells are mainly unknown however. As T lymphocytes are among the dominant cell typs in psoriatic lesions effects of fumaric acid derivatives on these cells were investigated. Human peripheral CD 3+ T lymphocytes were obtained from buffy coats of healthy donors by Ficoll Hypaque centrifugation and the sheep erythrocyte rosetting technique. Cell purity as controlled by FACS analysis exceeded 95 %. Cells were then incubated with in with different concentrations of 8 fumaric acid derivatives (1. methylhydrogenfumarat, 2. dimethylfumarat, 3. ethylhydrogenfumarat (Mg-salt), 4. ethylhydrogenfumarat (Ca-salt), 5. ethylhydrogenfumarat (Zn-salt), 6. fumaric acid, 7. monoethylfumarat, 8. monomethylfumarat) in RPMI 1640 plus 10 % FCS. Half of the cells were *coincubated with 50 ng/ ml phorbol myristrate acetate (PMA).To all wells 20 µl of Alamar Blue, a* metabolic activity indicator, were added in the beginning of the incubation time. The plates were then incubated for up to 5 days and metabolic activity was assessed every 24 hours by fluorometric measurements. At the same time points aliquots of cell supernatants were taken to determine the concentrations of secreted RANTES and IL-8, as tested by sandwich ELISA. Only four of the assessed derivatives (no. 1,2,5,8) gave a dose dependant suppression of the metabolic activity, which was seen as early as 24 hours post incubation and was constant over the whole test period. The suppressive effect was more pronounced for those cells which had been coincubated with PMA. In respect to RANTES secretion the addition of fumaric acid derivatives did not alter the amount of secreted chemokine in unstimulated cells, whereas in PMA stimulated cells distinct differences between the 8 derivatives were seen. Derivative no. 2 had a strong, dose dependant negative influence on the secretion of RANTES, whereas no. 1 and no. 6 enhanced the secretion. A similar increase of the IL-8 secretion was seen for no. 7 and no. 8. These results give evidence for distinct differential effects of fumaric acid derivatives on T lymphocytes in vitro and partially explain the therapeutic effects of these drugs as seen in the treatment of psoriasis.

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FUMARATES INDUCE IL-10 AND INTERFERE WITH THE PSORIATIC CYTOKINE NETWORK. Ockenfels HM, Kleber G, Funk R, and M. Goos. Dept. of Dermatology, University of Essen, Essen, Germany.

The "fumaric acid therapy" has been shown to be very effective in the treatment of patients with psoriasis, but the immunologic mechanisms of the complex mixture of fumaric acid derivatives (FADs) in psoriasis are still unclear. Therefore we determined if FADs influence the cytokine production of T-cells and/or keratinocytes (Kc) using our recently described co-culture model with Kc and HUT 78 T-cells. HUT 78 T-cells are capable to activate keratinocytes without cytokine-pretreatment and are producing T_H1 as well as T_H2 cytokines.

Mono-cultures and co-cultures of keratinocytes from psoriatic patients (n=9) and from controls (n=12) as well as HUT 78 mono-cultures were treated with various *concentrations of dimethyl-fumarate (DMF), zinc-monoethylfumarate (MEF),* calcium-MEF and magnesium-MEF for 24 hours. After the incubation period, the culture supernatants (sns) were analyzed for cytokine content of IFN-γ, Il-6, Il-8 and Il-10 using ELISA-technique, and the number of cells were determined. All four FADs provoked elevated levels (>20%) of Il-10 in the psoriatic as well as in the control co-cultures. Highest Il-10 induction was determined in calcium-MEF (10 μM) and magnesium-MEF (100 μM) treated psoriatic co-cultures (161% and 143%, respectively). In contrast, IFN-γ was diminished by all FADs in HUT 78 cultures as well as in co-cultures by 10%-40%. Furthermore, Il-6 was reduced only in psoriatic mono- and co-cultures by 20% (most prominent by DMF and zinc-MEF), but not in mono- and co-cultures from controls. The Il-8 secretion was not influenced by FADs.

The induction of the T_H2 cytokine Il-10 in co- and HUT-78 mono-cultures and the specific inhibition of Il-6 in psoriatic co-cultures could be also a hint for therapeutic mechanisms of fumaric acids in the treatment of psoriasis in vivo.

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EFFECTS OF TOPICALLY APPLIED CAPSAICIN IN ATOPIC ECZEMA

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Alterations in the cutaneous vascular system are prominent in atopic eczema. Although itching is the cardinal symptom of atopic eczema, its pathogenesis remains to be explained. We evaluated the effects of topically applied capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), a known inhibitor of cutaneous vasodilatation, on cutaneous reactions, alloknesis and itching in 12 patients suffering from atopic eczema and 12 healthy volunteers. Dolenon® (Capsaicin 0,05%) was applied three times daily over a five-day-period on the same infrascapular region. Vasocutaneous reactions and the antipruritic effect on subsequent histamine iontophoresis (20 mC) were tested on the following day. The results were compared to pretreatment with a placebo substance and to native skin. Wheal and flare areas were planimetrically evaluated. Itching, burning and stinging were rated every minute over a 24-minute-period on a scale. The examination also comprised alloknesis (=itchy skin), i.e. elicitation of perifocal itch sensation by usually non-itching (e.g. mechanical) stimuli. In controls Dolenon® pretreatment significantly reduced flare sizes compared to atopic eczema patients. In contrast to controls, atopics showed a total lack or significantly smaller areas of alloknesis. Dolenon® pretreatment reduced itch sensations significantly compared to non-pretreated skin and basic substance in controls but not in atopic eczema patients. Atopics rated significantly higher itch intensities in Dolenon® pretreated skin compared to controls. Burning, stinging or redness of the skin was reported by all subjects on initial application of Dolenon®. We conclude that Capsaicin 0,05% can be an effective antipruritic substance in healthy skin but not in patients suffering from atopic eczema. These results further imply that in atopic eczema Capsaicin has a diminished influence on vasocutaneous reactions.

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INCREASED PRODUCTION OF GM-CSF BY KERATINOCYTES MAY FAVOR SUSTAINED DENDRITIC CELL ACCUMULATION AND ACTIVATION IN ATOPIC DERMATITIS SKIN.

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Atopic diseases are characterized by important dysregulation in the expression and release of cytokines involved in the control of both inflammatory and immunologic responses. In order to examine the possible contribution of epidermis to the pathogenesis of atopic dermatitis (AD), keratinocyte (KC) cultures were established from uninvolved forearm skin of patients with severe AD and from healthy subjects, and their response to the action of a variety of stimuli was compared in terms of cytokine production. Second- or third-passage cultured atopic KC exhibited a higher spontaneous GM-CSF release compared to KC from healthy controls. Treatment with PMA (1-10 ng/ml) stimulated a GM-CSF release which was 5-10 fold-higher in atopic KC compared to control KC. In parallel, RT-PCR analysis showed a higher constitutive as well as PMA-induced GM-CSF gene expression in AD-derived KC. On the other hand, PMA did not cause any differentiated response in the secretion of IL-1α, TNF-α, or IL-10. IL-1α (50 ng/ml) also promoted higher GM-CSF secretion in atopic than in control KC, whereas LPS (1-50 μg/ml), lipoteichoic acid (1-50 μg/ml), SEB (50-500 ng/ml), TSST-1 (50-500 ng/ml) or TNF-α (150 U/ml) did not induce significant GM-CSF release in both control and atopic KC. Presence of hydrocortisone (0.4 μg/ml) in the culture medium strongly reduced GM-CSF release from both atopic and control KC, and the downregulation was also visible at the mRNA level. Finally, supernatants from untreated or PMA-treated atopic KC were able to strongly stimulate PBMC proliferation, and this effect was inhibited by anti-GM-CSF mAb. A higher production of GM-CSF by atopic KC may be relevant to the increased recruitment, sustanied activation and enhanced antigen-presenting-cell functions of dendritic cells in AD skin.

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REACTIVITY AGAINST HOUSE DUST MITE ANTIGENS AND LEVELS OF MITES IN THE BEDS OF PATIENTS WITH ATOPIC DERMATITIS.

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Some patients with atopic dermatitis (AD) have positive patch tests against house dust mite (HDM) antigens. Purified HDM antigen Der pI, however, exhibit a proteolytic enzyme activity comparable to serine proteinases. It is therefore likely that HDM's, apart from allergic reactions, also cause irritant reactions in atopic skin. Cutaneous reactivity against the highly purified HDM antigens Der pI and Der pII and the enzymes papain and trypsin was investigated in 36 unselected consecutive patients with AD. Twelve patients had type I allergy to HDM and of these, two had a true allergic type IV reaction towards the purified HDM antigens. Positive epicutaneous reactions were observed in another four patients, but this was combined with irritant reactions to papain and trypsin.

We also wanted to determine whether patients with AD (n=49) are more heavily exposed to HDM in their beds than patients with psoriasis (n=11) (chosen as another scaling skin disorder) and healthy controls (n=10).

We found no difference between the groups concerning the total amount of dust. Interestingly, the amount of dermatophagoides farinae was higher in dust recovered from the beds of patients suffering from psoriasis, when compared to AD and controls ($p < 0.05$). No significant differences were observed regarding the levels of dermatophagoides pteronyssinus and dermatophagoides microseras. No difference was observed in the IgE levels comparing patients with and without type I allergy to HDM.

In conclusion these observations indicate that the house dust mite may function not only as an allergen, but also as an irritant in patients with atopic dermatitis. Further, are patients with psoriasis more heavily exposed to dermatophagoides farinae, than patients with AD and normal healthy controls.

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mRNA INTERLEUKIN-10 EXPRESSION BY *IN SITU* HYBRIDIZATION IN ATOPIC DERMATITIS. IMMUNOLOGICAL SIGNIFICANCE.

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Interleukin (IL)-10 is produced by B and T lymphocytes, monocytes and keratinocytes. IL-10 plays a major role in suppressing immune and inflammatory responses by inhibiting the production of Th1 cytokines. Atopic dermatitis (AD) provides an environment for the development of a Th2 secretion pattern, not only for IL-4 and IL-5 but also for IL-10. The aim of this study is to evaluate mRNA expression (exp.) by *in situ* hybridization of IL-10 in chronic and acute AD and to compare it after the treatment with diet supplements rich in linoleic acid (LA). Samples were obtained from AD patients, 22 chronic eccema (Rajka score ≥7) and from 8 positive patch test to aeroallergens (Der pTI and Der Fa 40μg/ml, 20μl). Cutaneous samples from the same AD patients with chronic eccema were evaluated after LA intake (3gr/day, 6 weeks) (n=10). Biotin IL-10 oligonucleotide (800 ng/sample) from R&D Systems and 5μg/ml proteinkinase was employed. Internal control of mRNA preservation was PolydT. The best positive and negative control of mRNA IL-10 exp. were malignant melanoma and contact dermatitis. Healthy skin individuals were also studied (n=5). mRNA IL-10 exp. in healthy skin was negative. 18 samples of chronic and severe AD showed an intense mRNA IL-10 exp., 2 samples were loose and 2 samples were negative. 14 samples of chronic AD showed a diffuse and strong mRNA IL-10 exp. on inflammatory infiltrate and keratinocytes and 4 only in the inflammatory infiltrate. All this samples were negative without probe. mRNA IL-10 exp. was negative in all positive aeroallergen patch test and after LA intake. We conclude that mRNA IL-10 is the best Th2 cytokine expressed by *in situ* hybridization in chronic and severe AD. Previously, we demonstrated IL-4 and IL-5 *in situ* mRNA exp. in positive patch test by aeroallergen. Negative mRNA IL-10 exp. after LA intake correlates well with clinical and pathological improvment. IL-10 exp. could explain the difficulty of atopic patients to develop contact dermatitis.

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RELATIONSHIPS BETWEEN INTERLEUKIN 4 PRODUCTION AND CD23 EXPRESSION IN THE LESIONAL SKIN OF PATIENTS WITH ATOPIC ECZEMA

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We have previously reported the abberant expression of the low affinity receptor for IgE [CD23] on antigen presenting cells [APC's] in the lesions of atopic eczema. *In vitro* studies have implicated the cytokine IL4 in regulating levels of CD23 expression. To test the relationship between IL4 and CD23 *in situ* , IL4 mRNA and CD23 expression were analysed in lesional skin before and after efficacious therapy. mRNA for IL2 was also investigated.

4mm punch biopsies were removed from eczematous lesion skin before and after 2 months treatment with Chinese Herbal Therapy [Normal skin was also biopsied as a control]. Efficacy of therapy was determined by recording scores for erythema on clinical examination at both time points. Frozen sections of the biopsies were used to reveal cytokine mRNA [using *in situ* hybridisation] and CD23 expression on antigen presenting cells [using immunocytochemical techniques]. Levels of expression were quantified using computerised image analysis systems recording optical density of reaction product.

The results revealed raised CD23 expression [OD 0.48 lesional skin,0.12 normal skin] before treatment and raised expression of mRNA for IL4 [OD 0.24 lesional skin and 0.14 normal skin] before treatment. Following treatment both CD23 expression and IL4 mRNA were reduced to near normal levels [OD CD23 0.27 OD IL4 mRNA 0.18]. No significant reduction in mRNA for IL2 was observed after treatment. However pre-treatment levels were reduced compared to normal and these showed a modest rise after treatment.

This data supports the hypothesis that an association between IL4 production and CD23 expression exists *in situ* in eczematous lesions; and further implies a possible shift from TH2 to TH1-like T cell activity following efficacious therapy.

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ALLERGEN-SPECIFICITY OF SKIN-INFILTRATING T-CELLS IS NOT RESTRICTED TO A TYPE 2 CYTOKINE PATTERN IN CHRONIC SKIN LESIONS OF ATOPIC DERMATITIS.

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The majority of allergen-specific T cells derived from inhalant allergen patch test lesions in patients with atopic dermatitis were previously found to produce a restricted type 2 cytokine pattern. Recent studies, however, have revealed that in chronic eczematous skin lesions of patients with atopic dermatitis, expression of the type 1 cytokine IFN-γ predominates. In order to evaluate cytokine production by allergen specific T cells in chronic atopic dermatitis, house dust mite (Dermatophagoides pteronyssinus) specific T cell clones were established from the dermis of chronic skin lesions of sensitized adult patients with atopic dermatitis. Frequencies of skin-derived T cells proliferating in the presence of Dermatophagoides pteronyssinus were between 1/138 and 1/4255 indicating that only a minority of skin-infiltrating T cells is allergen-specific. When these cells were analyzed for their capacity to produce IFN-γ, the majority, that is 71% of these cells, were found to express IFN-γ mRNA and to secrete IFN-γ protein, either alone or in combination with IL-4. Phenotypic analysis revealed that 15% of skin-infiltrating allergen specific T cells were $CD8^+$. No selection of Vβ elements was detected in Dermatophagoides pteronyssinus specific T cell clones. These studies demonstrate that allergen-specifity of skin-infiltrating T cells is not restricted to a type 2 cytokine pattern in lesional atopic dermatitis. The notion that the majority of allergen-specific, skin-infiltrating T cells is capable of producing IFN-γ further supports the concept that IFN-γ expression is of major pathogenetic relevance for the chronic phase of atopic dermatitis.

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PATIENTS WITH ATOPIC DERMATITIS HAVE AN ELEVATED LEVEL OF SOLUBLE CD30

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The 120 kDa CD30 surface antigen is expressed by a subset of activated T and B lymphocytes. CD30 also has an 88 kDa soluble form (sCD30). The purpose of this study was to analyze the serum levels of sCD30 in patients with atopic dermatitis (AD), and to establish the possible correlation between the concentration of sCD30 and the levels of total serum IgE. Serum from 19 AD patients, median age 30 years (range 20-64 years), and 20 non-atopic age- and sexmatched healthy controls were analyzed for the presence of sCD30. The AD patients had a median serum IgE level of 680 kU/l (range 24-7900 kU/l), while it in the healthy controls was 36 kU/l (range 3-83 kU/l). The sCD30 levels in serum was measured with a commercial ELISA test kit (Dakopatts, Copenhagen, Denmark). It was found that the sCD30 levels in the AD patients were significantly higher (p=0.018, Mann-Whitney rank sum test) compared to the non-atopic healthy controls; median 45 U/ml (range 4-230 U/ml) versus median 15 U/ml (range 4-140 U/ml). The sCD30 levels were not related to the levels of total serum IgE. These data support the hypothesis that patients with AD have elevated levels of sCD30. However, there does not seem to be any correlation between the concentration of sCD30 and the total serum IgE levels.

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DECREASED INTERFERON-γ-PRODUCING CD4+ CELLS IN PATIENTS WITH ATOPIC DERMATITIS MEASURED AT THE SINGLE CELL LEVEL.

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Recently decreased interferon(IFN)-γ and increased interleukin(IL)-4 production have been reported by measuring the content of the cytokines in culture supernatants of peripheral blood mononuclear cells(PBMC) from patients of atopic dermatitis(AD). These data suggest deviation of PBMC into Th_2-reactive cells in AD. In the present study, we examined the frequency of IL-2-, IL-4-, and IFN-γ-producing cells in PBMC at the single cell level. PBMC from 10 patients with AD and 9 controls were stimulated for 2 days with anti-CD3 and IL-2, and cultured with the maintenance dose of IL-2 for further 4 days. Cells were then restimulated with PMA and ionomycin for 4 hours in the presence of monensin. After fixation of the cells, the cell membranes were made permeable and intracellular cytokines were stained togeter with anti-CD4 or CD8 antibodies. Cytokine-producing cells were analyzed by gating $CD4^+$- or $CD8^+$-subsets. IFN-γ-producing cells were decreased (12±5.4%) in $CD4^+$ subsets of AD patients when compared with those in the controls (28±14%). There were no significant differences in IL-2- or IL-4-producing $CD4^+$ subsets or in $CD8^+$ subsets. These results suggest a role of IFN-γ-producing $CD4^+$ cells in the pathophysiologic state of AD.

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DIFFERENT PHENOTYPES AND CYTOKINE PATTERN IN FOOD SPECIFIC T LYMPHOCYTES FROM CHILDREN AND ADULTS WITH FOOD RESPONSIVE ATOPIC DERMATITIS.

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In this study lymphocyte responses to food-antigens from children (median age: 3.3 years, range 0.7 - 5.9 years) and adolescents/ adults (median age: 29.2 years, range 17.3 to 47.0 years) with food responsive atopic dermatitis were compared. These patients reacted to double-blind, placebo controlled oral food challenge (cow milk and/or hen's egg) with a deterioration of AD. The proliferative response of food-allergen stimulated lymphocytes were in the same range when children and adults were compared. In contrast, the frequencies calculated from limiting dilution cultures of T-cells proliferating in response to casein or ovalbumin were markedly lower in sensitized children (mean: 1/1389) as compared to adult patients (mean: 1/309). 81 T cell clones (TCC) were established from the blood of these patients. The majority (64%) of food antigen specific TCC from adults but only 43% of casein- or ovalbumin-specific TCC from children were $CD4^+$. All TCC produced high amounts of IFN-γ upon stimulation with concanavalin A. Additional IL-4 secretion was detected in the majority of TCC derived from children and not from adult patients. These results indicate: (i) Food-specific proliferation of blood lymphocytes can be detected both in children and adults with food responsive AD, although the frequencies of specific circulating lymphocytes appear to be notably lower in children with clinically relevant food allergy upon stimulation with specific allergens in vitro and (ii) both the phenotype and cytokine pattern of food specific T-cells are different in sensitized children as compared to adolescents/adults with atopic dermatitis.

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TOPICAL TACROLIMUS (FK-506) TREATMENT LEADS TO PROFOUND ALTERATIONS OF THE ANTIGEN PRESENTING CELLS IN LESIONAL ATOPIC DERMATITIS SKIN.

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Tacrolimus (FK-506) has recently been shown to be an effective topical drug for atopic dermatitis (AD). However, little is known about the mode of action on the cellular level. Therefore, ex-vivo studies were conducted in 10 AD patients taking part in a clinical trial with FK-506 ointment. Single epidermal cell (EC) suspensions were prepared from treated and untreated AD lesions for quantitative flow cytometric analysis and skin mixed lymphocyte reactions (SMLR). Untreated lesional AD skin contained high numbers of $FcεRI^{++}$, $CD1a^+$, Birbeck granule negative Inflammatory Dendritic Epidermal Cells (IDEC) but low numbers of $FcεRI^+$, $CD1a^{++}$, Birbeck granule positive 'classical' Langerhans cells (LC). FK-506 treatment clinically improved the skin lesions, reduced the numbers of IDEC, but augmented the percentage of LC. Furthermore, FK-506 treatment downregulated the FcεRI-expression in both the LC and IDEC populations. Moreover, analysis of treated and untreated EC in the SMLR revealed an 80±12% reduction of the stimulatory capacity of treated EC to their autologous lymphocytes by topical FK-506 treatment. We conclude that FK-506 is an effective immunomodulatory drug for AD, qualitatively, quantitatively and functionally influencing the inflammatory cellular infiltrate whilst leading to clinical remission of the disease.

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AGE-DEPENDENT DIFFERENCES OF HISTIDINE-, UROCANIC ACID AND UROCANIC ISOMERS CONCENTRATION IN ATOPIC ECZEMA.

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In atopic eczema (AE) an impairment of the endogenous UV filter system has been shown recently. A possible correlation between disease severity and parameters of the endogenous filter system has not been looked at so far.

Concentrations of UCA, its *trans*- and *cis*-isomers and the UCA precursor, histidine were analysed by HPLC from skin eluats won under standard conditions. 32 patients with AE (1-81 years) and 10 healthy non-atopic controls (3-42 years) were included. Skin sampling was performed in all subjects on the abdomen, the back and both thighs. In addition, in patients with AE the two most severely affected sites were sampled and graded using three criteria (eryth., excoriat., lichenif.), each on a 0-3 scale. Results confirmed earlier data of reduced UCA-concentrations in patients with AE. A correlation between disease activity and any of the test parameters could not be found. The *trans*-UCA/histidine ratio which characterises the histidine activity was significantly reduced within the group of patients with AE (0.65 versus 0.96) indicating a diminished tranfer rate from histidine to *trans*-UCA. In addition, this ratio was age-dependent. In the group of AE patients aged between 1 to 20 (n=14) the ratio was 0.75 for all test sites with a ratio of 1.01 for the unaffected skin and a ratio of 0.59 for affected skin sites. The corresponding data for patients aged > 20 (n=18) were: 0.60 for all skin sites, 0.56 for diseased skin and 0.66 for uninvolved skin. Data give further evidence for a histidase dysfunction in AE. It might be speculated that in younger patients the enzyme concentration or capacity is sufficient for maintenance of physiological UCA levels in uninvolved skin (*trans*-UCA/histidine-ratio unchanged from the non-atopic control group) with a loss of this later on (reduced *trans*-UCA/histidine-ratio in comparison to the control group).

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AVENE SPRING WATER REDUCES INTESTINAL PERMEABILITY TO LACTULOSE IN ATOPIC PATIENTS.

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Anecdotal observations on remission of Atopic Dermatitis (AD) after intake of low salt water, and conversely AD exarcerbation with oral calcium supplements have recently been reported. We here investigated the effects of an oral diet with the weakly mineralized Avene spring water in AD patients with increased intestinal permeability to food allergens. 51 AD patients of at least 10 years of age who undertook a water cure in Avene, France, were screened for participation to the study. Screening procedure consisted of measuring lactulose (Duphalac®, Duphar Laboratories), as a reflectance of increased intestinal permeability to food allergens, and mannitol (Aguettant Laboratories) used as control, in urine of patients during the five hours after ingestion of 5 g of each marker. The volume of urine collected was measured after homogenization, and clearance of lactulose and mannitol calculated after determination of the sugars by gas-liquid chromatography. Patients with abnormal levels of lactulose (> 0.6 %) entered the study (baseline). During the treatment course of 18 days, enrolled patients were asked to drink exclusively Avène water (≥ 1 500 ml/day). Patients were also asked not to modify their usual food regimen. Systemic antiinflammatories and antihistamines, as well as systemically absorbed topical corticosteroids were not permitted. At the end of treatment (day 18), sugar ingestion, urine sampling and dosage procedures were repeated. Out of 51 AD patients screened, 7 patients were enrolled because of abnormal levels of urine lactulose at baseline (mean 1.167 % ± 0.253 SD). At the end of treatment (day 18), lactulose concentration in urine diminished dramatically by a mean change of 0.603 % (mean 0.564 % ± 0.292 SD, $p < 0.01$). Urine mannitol was found to be normal and constant over the cure period. In conclusion, Avene water taken orally improves significantly one of the hallmark of food intolerance in AD patients, i.e lactulose intestinal permeability. Whether its action is due to its poor mineralization, its high content in silicates or to other factors remains to be determined.

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Nonanoic acid induces apoptosis in epidermal Langerhans cells.
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We compared proliferation and apoptosis in early (1 and 6 hrs) and late (24 and 48 hrs) irritant reactions and uninvolved skin from 47 patients with chronic irritant contact dermatitis. Irritants were 80% nonanoic acid (NA) and 5% sodium lauryl sulphate (SLS). Basal and prickle layer apoptosis was determined in H&E sections and confirmed using TUNEL. Epidermal proliferation was demonstrated by staining for the Ki-67 nuclear antigen. Sections were scored blindly for apoptotic or proliferating cells.

NA decreased the numbers of proliferating cells by 6 hrs which then increased above normal by 48 hrs. SLS showed a trend towards increased proliferation by 48 hrs. The prickle layer showed increased numbers of apoptotic cells by late timepoints after both NA and SLS. In contrast only NA induced basal layer apoptosis at both early ($p<0.03$) and late ($p<0.04$) timepoints. The balance between basal layer cell death and proliferation is thus distinct between the two irritants.

Significantly, one third of basal layer and half of prickle layer apoptotic cells seen after NA application were also CD1a+ve. SLS failed to induce apoptosis in CD1a+ve cells. **To our knowledge this is the first description of CD1a+ve Langerhans cell apoptosis *in situ* induced by an epidermal irritant.**

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INDUCTION OF APOPTOSIS IN HUMAN DERMAL MICROVASULAR ENDOTHELIAL CELLS (HDMEC) BY IFNα - A POSSIBLE EXPLANATION FOR THE INVOLUTION OF HAEMANGIOMAS AFTER IFNα TREATMENT.
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Haemangioma of infancy is an angiomatous disorder characterized by the proliferation of capillary endothelium. It has recently been shown that IFNα may induce involution of proliferating, life-threatening haemangiomas in children. This IFNα induced regression of haemangioma is not accompanied by any T-cell response or by the occurrence of necrosis. Therefore we investigated *in vitro* if apoptosis may be induced by IFNα using HDMEC. HDMEC were stimulated at different time points with IFNα at concentrations of 100, 500, 1000 and 2000U/ml. Apoptosis was detected by *t*erminal deoxynucleotidyl transferase-mediated FITC-d*U*TP *n*ick *e*nd-*l*abeling (TUNEL) (Sgoonc R, et al TIG 10:14,1994). Quantitative analysis was performed using the FACScan; morphological alterations were studied by confocal laser scanning microscopy. Apoptosis was detected in up to 20% of the stimulated endothelial cells compared to the untreated cells. A maximum of apoptosis was observed after 48 hours of stimulation with IFNα in a dose dependent manner. Although investigations on umbilcial vein endothelial cells revealed inhibition of proliferation and a possible downregulation of production of angiogenic growth factors was discussed as a possible therapeutic effect of IFNα, the mechanism is still unknown. Our data show for the first time that IFNα induces apoptosis in HDMEC. This *in vitro* finding might explain the *in vivo* effect of IFNα on hemangioma with clinically and histologically observed involution without any sign of inflammation or necrosis.

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CD40 TRIGGERING REDUCES ULTRAVIOLET B-INDUCED APOPTOSIS IN HUMAN EPIDERMAL LANGERHANS CELLS.
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We have recently shown that *in vitro* low-dose of ultraviolet B radiations (UVB, 100 to 200 J/m^2) directly impaired the antigen presenting function of human Langerhans cells (LC). While UVB irradiation at 100 J/m^2 did not impair LC viability, higher dose (200 J/m^2) significantly reduced the number of viable LC after 2 days of culture. Furthermore a large number of viable LC was enlarged and had lost their characteritic dendritic morphology. In this study, we showed that UVB-induced decreased LC viability is due to apoptotic cell death. As shown by electron microscopy, apoptotic cells displayed typical morphological changes such as chromatin and cytoplasm condensation, endoplasmic reticulum dilatation and membrane blebbing. Using Hoechst 33342 staining, apoptotic cells with condensed nuclei were clearly recognized and enumerated. After two days of culture, about 10-20% of unirradiated purified LC had condensed nuclei. By contrast, the percentage of apoptotic cells reached 50-70% in LC suspensions irradiated at 200 J/m^2. We then examined the effect of CD40 triggering on UVB-induced apoptosis. To this end, LC were cultured for 2 days on CD40 ligand transfected L cells (CD40Lc) or CD32 transfected L cells (CD32c) as controls. As previously described, CD40 cross-linking improved the viability of unirradiated LC after a 2-day culture. After irradiation at 200 J/m^2, viable LC recovery was significantly higher in CD40 activated LC than in controls. In addition, CD40 triggering efficiently reduced the number of apoptotic cells in of irradiated LC suspensions.
Collectively, these results demonstrate that *in vitro* exposure to high dose UVB radiations induces LC apoptosis and that CD40 triggering partly prevents this phenomenon.

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APOPTOSIS IN THE MORPHOLOGY OF THE LYME DISEASE.
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There are a lot of morphological changes described in the skin tissue of Acrodermatitis chronica atrophicans (ACA) and Erythema migrans (EM) previously. The dermis is believed to be the most involved skin layer, however, very little information is available on its epidermal part. This was the goal of the present work.
Skin biopsies of twenty patients with a typical clinical course and positive serology of Lyme disease were examined by using light and electron microscopy.
Vacuolar degeneration and dark shrinked nuclei mainly in the lower part of epidermis could be seen on the light microscopical picture which confirmed the diagnosis of apoptosis. Beside keratinocytes, both Langerhans cells and melanocytes were mainly recognized as apoptotic cells. Borrelia was found in blebs of these cells and in intercellular spaces. No other abnormalities of the epidermal structure were observed which corresponded also with a lack of the inflammatory infiltration.
The damage of the Langerhans cell as an antigen presenting cell might be one step of the quick reaction inducing apoptosis in the epidermis. Further experiments which are in progress at our Department should be performed to confirm the above mentioned hypothesis.

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TRANSFORMING GROWTH FACTOR-β, 1,25-DIHIDROXYVITAMIN D3, CALCIUM AND ULTRAVIOLET B RADIATION, BUT NOT TUMOR NECROSIS FACTOR-α INDUCE APOPTOSIS IN CULTURED HUMAN KERATINOCYTES.
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Apoptosis seems to play a major role in the maintenance of epidermal homeostasis and in the mechanisms of hyperproliferative and neoplastic disorders. In addition, apoptotic keratinocytes (K) have been detected in normal human skin. The purpose of the present study was to evaluate the induction of apoptosis upon several stimuli in normal human K in culture. K were cultivated in serum-free medium with or without transforming growth factor-β (TGF-β, 30 ng/ml), calcium (Ca++, 2 and 3 mM), 1,25 dihidroxyvitamin D3 (D3, 1μM) and tumor necrosis factor-α (TNF-α ,500U/ml). Furthermore, K were irradiated with increasing doses of ultraviolet B radiation (UVB, 10, 25, 50, 75 mJ/cm^2). K were collected at different times and apoptosis assessed by TUNEL technique. Because this allows quantitative evaluations, apoptosis was time and dose-dependently studied. TGF-β induced apoptosis at 120 and 144 hrs, with 30% TUNEL positive cells. Ca++ induced a dose-dependent increase of apoptotic K from 12 hrs up to 36 hrs, as compared to controls ($p<0.001$). After treatment with D3, 30% apoptotic K were observed at 144 hrs. Finally, UVB-induced apoptosis was shown to be dose-dependent and a significant number of TUNEL positive cells were detected even at low doses (10mJ/cm^2, $p<0.002$) as compared to sham-irradiated K. Apoptosis was confirmed by the appearance of DNA ladder on agarose gel electrophoresis and by electron microscopy. This study indicates that stimuli which are involved in the regulation of cell proliferation and differentiation can also induce K apoptosis.

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DOWNREGULATION OF BCL-2 BY ANTISENSE OLIGONUCLEOTIDES REDUCES TUMOR SIZE AND IMPROVES CHEMOSENSITIVITY OF HUMAN MELANOMA IN SCID MICE
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Malignant melanoma, a prime example for poor response to various treatment modalities including chemotherapy, expresses bcl-2 in up to 90% of all cases. The anti-apoptosis gene bcl-2 belongs to a new category of oncogenes capable of regulating programed cell death. Induction of programed cell death has been proposed recently as the mechanism of action for a variety of chemotherapeutic agents. In the present study we could show a sequence specific downregulation of bcl-2 protein by phosphorothioate antisense oligonucleotides in human melanoma *in vitro*. The levels of three control proteins remained unaltered. In addition, we demonstrate in a newly established SCID-hu xenotransplantation melanoma model (n=6-7 animals per group), that antisense oligonucleotides directed against bcl-2 are capable of causing a statistically significant decrease in tumor weight ($p<0.004$). Bcl-2 antisense treatment also improved the chemosensitivity of human melanoma rendering animals without detectable tumors after a combined bcl-2 antisense - dacarbazine treatment. Reverse controls and mismatch phosphorothioate oligonucleotides had no such effects. Our findings stress the notion that downregulation of bcl-2 in human melanoma may be a novel approach to improve chemosensitivity in this type of malignancy.

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LOSS OF BCL2 PRECEDES THE DECAY OF T CELL POPULATIONS IN RESOLVING MANTOUX REACTIONS

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T cell apoptosis is regulated in part by levels of BCL2 protein in the cytoplasm. The presence of this protein inhibits programmed cell death whereas loss of BCL2 expression signals the initiation of the apoptotic process. The Mantoux reaction is a self limiting dermal delayed type hypersensitivity reaction characterised by T cell infiltration which peaks at 3 days and then decays in 4-10 days thereafter. This study investigates the presence of BCL2 in the T cell infiltrates of this reaction and determines the relationship between BCL2 expression and the resolution of the lesion.

4mm punch biopsies were obtained at progressive times during a Mantoux reaction. At each time point erythema was recorded and the numbers of T cells infiltrating perivascular areas of the dermis and in the epidermis were quantified using immunohistology and image analysis on frozen sections. At all time points the proportion of T cells expressing BCL2 was quantified using double immunofluorescence staining.

The results revealed that the T cell perivascular infiltration peaked at 72 hours [18.2cells per unit area], and declined by day 14 to 9.9 cells per unit area. In the epidermis fewer T cells were seen [72 hours, 4.85cells per unit area], day 14 0.48 cells per unit area. Proportions of perivascular cells expressing BCL2 were initially 65% [at 6 hour time point] peaked at 24 hours [83.5%] and then progressively declined to 23% at day 14. In the epidermis 80% of T cells were BCL2 positive at 6 hours. This number progressively declined to 12.5% at day 14. In summary T cells infiltrating a man?? reaction express relatively normal levels of BCL2. This peaks in the dermal infiltrates at 24 hours and then declines to a very low level by day 14. It is concluded that loss of BCL2 possibly promoting apoptotic cell death is associated with a resolution of this cell mediated immune reaction in the skin.

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PROGRAMMED CELL DEATH IN HUMAN SKIN AS RELATED TO CALCIUM GRADIENTS: STUDIES USING THE LUND SCANNING MICROPROBE

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The distribution of elements and trace elements of skin cross sections have been mapped using a scanning nuclear microprobe. A conspicuous feature is a Ca-gradient that rises to relatively high levels in the str. granulosum and drops to values close to nil in the str. corneum. This corresponds to the finding from epidermal culture experiments that demonstrate a dependence of high Ca^{2+} levels for a formation of a complete str. corneum. In non-lesional psoriatic skin and in dry atopic skin the Ca-gradient is higher than that of normal skin. In addition, abnormally high Fe and Zn levels are recorded in the str. granulosum and corneum regions in the pathological skins which correlates to an increased cell turn over in the basal cell layer of the the psoriatic and atopic skins. The ratio of Ca/Zn in str. corneum of paralesional psoriatic skin is approximately 8:1 compared to 12:1 in normal skin and 15:1 in dry atopic skin. This underlines that the differentiation process in the paralesional psoriatic skin may actully be an example of disturbed programmed cell death, hence the finding of acantotic cells in this skin.

Our studies underline the feasibility of quantative analysis of physiologically important elements by particle probe analysis as a complement to immunological and biochemical methods in experimental dermatology.

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CLONING AND CHARACTERIZATION OF A NEW SPLICE VARIANT OF THE HUMAN BCL-X GENE.

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Proteins of the bcl-2 family are intimately involved in the regulation of cell survival by preventing apoptosis. In human keratinocytes, Bcl-x is the most predominant member of this family. Using high stringency DNA-DNA hybridization we have isolated a cDNA clone encoding a novel bcl-x homologue from a placenta cDNA library. The 5'-end of this cDNA is identical to the large form of Bcl-x, i.e. Bcl-xL, whereas no matches with known sequences were found for its 3'-end. The deduced amino acid sequence showed an aminoterminal part of 186 aa which is identical to Bcl-xL and contains the putative anti-apoptotic region of this family of proteins. The C terminal 39 aa are different from previously reported sequences of bcl-2 family genes. Sequencing of a genomic PCR product revealed that the novel cDNA was transcribed from the bcl-x gene without splicing. mRNA transcripts of this novel bcl-x variant are ≈ 3 kb long and were detected in various human cell types, including keratinocytes and melanocytes. Expression of the cDNA in bacteria produced a protein of about 27 kDa which reacted to an anti-bcl-x antiserum. The extensive identity between Bcl-xL and the novel variant of Bcl-x suggests that this novel splice form plays a role in the regulation of apoptosis.

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Inhibition of Neutrophil Granulocyte Apoptosis by Coculture of Human Microvascular Endothelial Cells. H. Smola, M. Reinke, S. Heß*, S. Anders, M. Hafner, T. Krieg and C. Mauch, Dept. of Dermatology and *Virology, University of Cologne, 50924 Cologne, FRG

PMN are considered to be the host´s first line of defense, yet, little is known about PMN life span and function after PMN have extravasated and can interact with activated dermal microvascular endothelial cells (DMEC), fibroblasts and keratinocytes.

Since endothelial cells are involved in inflammatory deseases we studied effects of coculture of DMEC on PMN apoptosis *in vitro*. Freshly isolated PMN entered apoptosis as demonstrated by condensed, hyperchromatic nuclei, DNA fragmentation and decrease of trypan blue excluding cells. Coculture of PMN with unstimulated DMEC increased PMN life span substantially evident by reduced DNA fragmentation and increase of viable cells. After 72 h controls (no DMEC) contained 7-12% trypan blue excluding cells, whereas coculture resulted in 60-70% viable PMN. Several cytokines were analyzed but only G-CSF, GM-CSF, IFN alpha had anti-apoptotic effects on control PMN (without DMEC). These cytokines were less effective i.e. reduced viable cells in cocultures compared with DMEC-PMN cocultures alone. Only IL 1 addition had a stimulatory activity on PMN life span in cocultures with no detectable effect on PMN alone. Since IL 1 seemed to act indirectly, DMEC were pre-stimulated with IL 1, washed several times and incubated with PMN in the absence on exogenous IL 1. Pre-treatment for 12 h was sufficient as well as conditioned medium to prevent PMN apoptosis. This indicates paracrine interactions of PMN and DMEC being modulated by IL 1 dependent seconday effects.

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CHARACTERIZATION OF CELL SIGNALLING VIA THE SPHINGOMYELIN CYCLE IN HUMAN KERATINOCYTES

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Sphingomyelin (SM), the most common sphingolipid, is an integral part of the lipid bilayer of the plasma membrane of keratinocytes. In the last few years, it has become obvious that SM takes part in a novel cell signalling pathway. The active intracellular messenger of the sphingomyelin cycle is ceramide which is the product of an agonist-stimulated sphingomyelin hydrolysis. Subsequently, increased levels of these breakdown products inhibit cell proliferation and induce cell differentiation and apoptosis in hematopoetic cell lines (1). In the present study, a time-dependent effect of *100 nM* 1α,25-dihydroxyvitamin D_3 and *30 nM TNFα on SM hydrolysis was* demonstrated in human keratinocytes. The hydrolysis of approximately 25 % of total cellular SM occurred after 3 h of 1α,25-dihydroxyvitamin D_3 treatment. In contrast to vitamin D_3, TNFα caused SM hydrolysis as early as 20 min after addition to the culture medium. This indicated the occurrence of early and late agonists of this signalling pathway. Using the ELISA and Northern blot techniques, an autocrine mechanism for the stimulation of SM hydrolysis by vitamin D_3 via TNFα expression was elucidated. *This signalling* pathway results in an apoptotic cell death of *human keratinocytes and* influences epidermal homeostasis.

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P53-INDEPENDENT EXPRESSION OF MDM-2 PROTEIN IN HUMAN SKIN.

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The MDM-2 proto-oncogene product p90 binds to and inactivates the P53 tumor suppressor protein. However, it remains to be seen if the unique role of MDM-2 is the regulation of p53 activity. In this work we have looked for MDM-2 expression both in normal human skin (NHS) and reconstituted skin (RS) at the protein level by *immunofluorescence using an anti-mouse monoclonal antibody (2A10) cross reacting* with human MDM-2 and at the mRNA level by in situ hybridization using a human antisense MDM2 RNA probe. Different p53 mutated keratinocytes cell lines were also studied. Results showed that MDM-2 protein is highly expressed in keratinocytes nuclei throughout the different layers of NHS, with increasing intensity from basal to superficial layers. The 2A10 monoclonal antibody recognized a 90 kD protein in human epidermal extracts that co-migrated with MDM2 present in 3T3DM murine cells used as control. In situ hybridization experiments detected a strong RNA signal in the epidermis *with higher intensity in basal cells. This indicate that MDM-2 expression in NHS could* be due to increased transcription of the gene, however, protein stabilization could contribute to the perduring high expression in superficial layers. Whereas, no MDM-2 protein was detected in the nuclei of keratinocytes in monolayers, detectable nuclear levels appeared early during epidermal reconstruction. Interestingly, wild-type P53 protein could not be detected simultaneously to MDM2 in all tissues and MDM-2 protein was also expressed in the P53 mutated keratinocytes cell lines. This is the first evidence for p53 independent expression of MDM-2 in a differentiated normal adult tissue, suggesting that it plays multiple functions in the cell, some of them being unrelated to the p53-MDM2 feedback loop.

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A FIRST INSIGHT INTO SIGNAL TRANSDUCTION BY TYROSINE PHOSPHORYLATION IN HUMAN EPIDERMAL LANGERHANS CELLS.

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Protein-tyrosine kinases (PTK) of the src- and syk-family are involved in many receptor-triggered signal transduction processes (e.g. FcεRI) resulting in events like cell growth, differentiation or immunological responses. Data about the expression of PTK's in human epidermal Langerhans cells (LC) are completely lacking. Using intracellular staining in flow cytometry and immunoblotting techniques, we could demonstrate the expression of $p72^{syk}$ and the src-family kinases $p60^{c\text{-}src}$, $p53/56^{lyn}$, $p59^{fyn}$, $p56/59^{hck}$ in LC of normal skin. In contrast, the PTK $p55^{c\text{-}fgr}$, which is highly expressed in the terminal differentiation stage of monocytes/macrophages, was not detectable. Furthermore, we found high levels of $p50^{csk}$, a PTK which regulates the activity of PTK's of the src-family. CD1a+ monocyte-derived dendritic cells showed a similar expression profile of these PTK's. In lesional skin of atopic eczema there was no correlation between the expression of strongly upregulated surface molecules like FcεRI on LC's and their potential signal transducing PTK. However, an increase in the expression of these PTK's was shown. CD1a+ inflammatory dendritic epidermal cells (IDEC) which immigrate *de novo* into lesional skin, showed a lower expression mainly of the src-kinases. In cultured normal LC, expression of these PTK's remained relatively constant. Addition of TNF-α, IL-4 or IFN-γ did not alter this expression profile. Neither spontaneous *in vitro* differentiation under GM-CSF nor the addition of IFN-γ could induce the expression of $p55^{c\text{-}fgr}$. In summary, we demonstrated that human LC express a large repertoire of receptor-associated PTK thus providing LC with multiple signal transduction pathways.

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A NEGATIVE CORRELATION BETWEEN BCL-2 AND P53 IN BASAL CELL CARCINOMA, AND AN APPARENT LACK OF P53 MUTATIONS IN NON-MELANOMA SKIN CANCERS FROM THE DUTCH POPULATION

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Dysfunctional p53 or constitutive bcl-2 expression can block a proper apoptotic response which is likely to facilitate cell transformation and tumor development. We have examined a series of non-melanoma skin cancers on the correlation between immunostainings for p53 and bcl-2 gene products. These data indicate that basal cell carcinomas, BCCs, and squamous cell carcinomas, SCCs, differ in the percentage of p53 immunopositivity. In addition, a significant negative correlation between p53 and bcl-2 expression indeed exists in BCCs, but not in SCCs. The lower frequency of spontaneous apoptosis in BCC seems to be strongly associated with bcl-2, while apoptosis in SCCs appears to be more prevalent, but not particularly related to p53 expression.

Direct sequencing of the exons 4-8 of the p53 gene yielded surprisingly few mutation (about 15% in SCC and BCC) when compared to the literature (90% SCC, 50% BCC in US, 50% in SCC in Sweden). Percentages of p53 mutations seem to shift with tumor type and geographical localization. Further studies are necessary in order to establish p53 mutations as a biomarker of skin tumor risk.

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EXPRESSION OF P21 AND P53 IN LANGERHANS CELL HISTIOCYTOSIS AND NORMAL LANGERHANS CELLS.

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Langerhans cell histiocytosis (histiocytosis X) is a clonal proliferative disorder of the dendritic antigen-presenting Langerhans cells (LCs) in the skin and other organs. The LCs show phenotypical features of aberrant activation and/or differentiation, but there is no pronounced proliferative activity of the cells, in spite of their strong expression of proliferation markers. To get insight into the molecular pathogenesis of these cellular alterations, we investigated the expression of the master switch gene p21, which encodes a cyclin-dependent kinase inhibitor that regulates the growth and differentiation of cells. Skin biopsies from 5 patients (3 infants, 2 adults) with Langerhans cell histiocytoses were incubated with moAbs against the p21 protein, the tumor suppressor gene p53, and the proliferation marker PCNA using light and electron microscopical immunolabelling. In all biopsies, the CD1a-positive tumour cells showed nuclear stainig for p21. This reaction was especially found in those tumour cells which demonstrated the morphologic features of cellular activation. The expression of p21 was paralleled by positive immunostaining for PCNA. In contrast, no expression of p53 could be observed in the tumour cells. In biopsies of normal skin (n=5), the CD1a-positive epidermal LCs expressed neither p21, nor p53 and PCNA. Taken together, the abnormal LCs of Langerhans cell histiocytosis, but not normal LCs express the p21 gene by a p53-independent pathway. This molecular pathway has very recently been shown to inhibit the PCNA-dependent proliferation and growth of cells by inducing their differentiation. Our data thus imply that the cellular behaviour of the LCs in Langerhans cell histiocytosis is due to a p21-mediated aberrant differentiation and activation of the PCNA-positive tumour cells.

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INVESTIGATIONS INTO THE CARCINOGENIC POTENTIAL OF TOPICAL COAL TAR.

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Recent publications have demonstrated polycyclic aromatic hydrocarbons in urine of patients using coal tar shampoos suggesting a carcinogenic potential for topical tar products. In this study we have investigated the carcinogenic potential of topical coal tar using a human skin explant system by assessing Langerhans cell (LC) function in a mixed epidermal cell/lymphocyte reaction (MELR) as a measure of immune surveillance and induction of p53 protein as a measure of nuclear damage.

50μl/cm^2 of 1, 2 and 5% solutions of coal tar in alcohol or emulsions in phosphate buffered saline and vehicle controls were applied to skin explants (6cm^2) using a template pinned to the explant and incubated at 37°C for 2 hours, washed and decontaminated . Part of the explant was processed for a standard MELR and the remainder cultured at 37°C for 20 to 24 hours before p53 was identified in epidermal lysates using Western blotting and staining with the mouse anti-human p53 antibody DO1.

5% coal tar preparations proved to be toxic to epidermal cells with up to 50% reduction in cell viability. Coal tar preparations caused a dose dependant reduction in LC function with significant reductions seen with both 1 and 2% preparations. 2% coal tar was seen to consistently upregulate p53 in epidermal cells.

The results of this study suggest that coal tar must be regarded as having a carcinogenic potential and safer therapeutic agents should be considered.

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CORRELATION BETWEEN ACTIVITY OF METALLOPROTEINASES AND MIGRATION ON COLLAGEN-I IN NORMAL AND TRANSFORMED HUMAN KERATINOCYTES.

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In vitro and *in vivo* investigations on various tumors have clearly shown a correlation between an increase in collagenase activities and neoplasia. The activation of signal transduction pathways by mutation or overexpression of cellular oncogenes has been associated with neoplastic transformation.

In our studies, we used primary cultured human keratinocytes, a human keratinocyte cell line spontaneously transformed, HaCaT, two HaCaT-ras clones metastatic or not obtained after H-ras transfection, and two cell lines isolated from cutaneous squamous cell carcinoma, SCL I and SCL II (Pr. Fusenig).

To assess migration mechanism, we performed locomotion assays by the method of " the phagokinetic track assay ". Our results showed that the migration index on collagen-I was similar for HaCaT cell line and normal cultured keratinocytes whereas it was higher for the HaCaT-ras and squamous cells.

The activity and the expression of collagenase A (MMP2) and collagenase B (MMP9) were studied by gelatin zymography and western blot analysis. The results showed that normal and transformed human keratinocytes expressed 72 and 92 kDa MMPs. The expression and activity of MMP9 were increased in HaCaT-ras and the carcinoma cells compared to HaCaT and normal human keratinocytes. The mutation of H-ras oncogene seems to play a primordial role in the motility of tumor cells by activation of MMP9. The mutation of p53 seems to have no effect because HaCaT and SCL II possessed a mutated p53 on the two alleles and their relative collagenase activities and migration were markedly different. The MMP9 induction may be related to an up regulation of AP1 transcription factor, whereas MMP2 expression, which is not regulated by AP1, is not modified. To confirm this hypothesis, the expression of MMP1, and tissue inhibitor metalloproteinase 1 and 2 (TIMP 1, TIMP2) are under investigation.

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RETROVIRAL-MEDIATED WILD-TYPE P53 EXPRESSION SUPPRESSES TUMORIGENESIS AND ALLOWS G1 ARREST AFTER UVB IN HUMAN SQUAMOUS CARCINOMA CELLS.

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p53 gene mutations in non-melanoma skin cancer argue for an important role of p53 in photocarcinogenesis. In order to investigate the function of p53 in this process, we performed retroviral-mediated gene transfer of wild-type p53 (wt-p53) into the human squamous carcinoma A253 cell line, lacking endogenous p53 expression. The effect of wt-p53 re-expression was investigated on the *in vitro* growth rate, tumorigenicity *in vivo*, differentiation status and cell cycle progression after UVB. In the p53-positive cells, tritiated thymidine incorporation was suppressed in the exponential growth phase by 30 to 60 %. Within 6 weeks following subcutaneous injection in nude mice, p53-negative cells formed massive ulcerating tumors, while p53-positive cells gave no tumors or very small tumors. The baseline mRNA expression of two precursors of the cornified envelope, SPR1 and SPR2, was increased 2-fold in the p53-positive A253 cells. Flow cytometry studies demonstrated that 5 h after 8 and 16 mJ/cm^2 UVB, only the p53-positive A253 cells showed an accumulation in the G1 phase, while at 24 h post-irradiation both the p53-positive and p53-negative cells exhibited a G2 delay/arrest. p21 mRNA was induced in a p53-dependent manner 5 h post-irradiation, consistent with its mediating role in this early cell cycle delay after UVB. We observed the same time course of p21 mRNA induction in human primary keratinocytes, making this approach of the p53 function relevant. We conclude that re-expression of wt-p53 in squamous carcinoma cells restores a behavior towards that of normal keratinocytes. Moreover this re-expression allows malignant keratinocytes to interrupt their cell cycle progression at the G1 phase, early after a biologically relevant UVB exposure.

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P53 EXPRESSION IN XERODERMA PIGMENTOSUM A CELLS WITH VARIOUS GENE MUTATIONS.

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p53 expression is induced by DNA damage including UV and plays important roles in DNA repair, cell cycle arrest and apoptosis. In XP group A cells, induction of p53 was reported to be remarkable, indicating that nuclear accumulation of p53 is related to DNA repair capacity. Analysis of XPA gene in Japanese patients revealed that most patients have homozygous mutations for the splicing site of intron 3 of the gene (AlwNI mutation). Other mutations are the nonsense mutation at codon 228 in exon 6 (HphI mutation) and at codon 116 in exon 3 (MseI mutation). Patients with HphI mutation have milder cutaneous and neurological manifestations due to the partially retained ability of repair. We questioned whether the p53 inducibility by UVB is different between XPA cells with various gene mutations. Further, the transcriptional activity of p53 in XPA cells was examined. By immunoblot analysis, fibroblasts of AlwNI homozygote and AlwNI/MseI compound heterozygote showed a marked induction of p53 by UVB irradiation compared with normal cells. Cells of AlwNI/HphI showed an intermediate reaction. p21 protein, which is transcribed in p53-dependent and -independent pathway, was expressed according to the p53 expression in XPA cells. These data suggest that p53 protein induction is dependent on the repair capacity of cells and induced p53 acts as a transcriptional factor.

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HIGH C-MYC GENE EXPRESSION POSITIVELY CORRELATES WITH METASTATIC POTENTIAL OF HUMAN MELANOMA IN SCID MICE

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Overexpression of the proto-oncogene c-*myc* and the oncogene N-*ras* have been associated with neoplastic transformation in a variety of tumors. We recently demonstrated that N-*ras* activation and overexpression does not contribute to metastatic potential of human melanoma in SCID mice. In the present study we newly established two SCID-hu xenotransplantation melanoma models overexpressing c-*myc*. Four c-*myc* transfectants (two clones per human melanoma cell line) and the appropriate vector controls were chosen for *in vivo* studies (n=7 animals per group). Interestingly, subcutaneously implanted melanomas overexpressing c-*myc* spontaneously formed metastases (lymph nodes, lung, liver) in all cases, wheras one vector control led to localized tumor growth only and the other failed to cause melanomas in the time period leading to terminal disease in animals injected with c-*myc* transfected human melanoma cells. C-*myc* is expressed in about 50% of all melanomas and small clinical studies found an association with advanced Clark levels and poor prognosis. However, no experimental evidence has been available to date and detailed investigations have been hampered by the lack of suitable animal models. Our findings stress the notion that levels of c-*myc* expression in human melanoma positively correlate with metastatic behavior and c-*myc* may therefore be an important prognostic marker for this type of malignancy.

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DIFFERENTIAL EXPRESSION OF p21 PROTEIN AND OTHER RELATED CELL CYCLE REGULATING PROTEINS IN EPITHELIAL SKIN CANCER.

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Several lines of evidence link aberration of the cell cycle to the development of cancer. A continuing enlarging group of cell cycle proteins namely cyclins, cyclin-dependent kinase (CDKs) and cyclin-dependent kinase inhibitors (CDIs) has been identified in the last two years. The present study was undertaken to evaluate the involvement of these proteins in epithelial skin tumours. We have examined immunohistochemically the expression of bcl-2 protein, p53 protein and related proteins(p21, MDM2), cyclin D1, CDKs(CDK4, Cdc2) and CDIs (p15, p16, p27) in 10 basal cell carcinomas(BCCs), 10 squamous cell carcinomas(SCCs) and 5 keratoacantoma(KAs). The expression data was then correlated with the proliferative index as measured by MIB1 antibody. A nuclear positivity for each reagents gave differents values for any kind of skin tumours analysed. BCCs:MIB1(15%), p53(10%), p21(3%); KAs: MIB1(40%), p53(30%), p21(20%); SCCs: MIB1(50%), p53(45%), p21(40%). No detectable signal was identified for cyclinD1, and CDIs proteins. Interestingly bcl-2 expression was only observed in BCCs cases. Our results indicate that in BCCs p21 protein is down regulated while in SCCs shows a quite similar reactivity of MIB1, p53 and p21. These data confirms previously in vitro experiments that demonstrated the capacity of bcl-2 to down regulate p21, a nuclear protein that play a key role in senescence and cellular differentiation.

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RAS MUTATIONS IN HUMAN MELANOMA ACTIVATE MIGRATION, SURVIVAL AND PROLIFERATION PATHWAYS ESSENTIAL FOR TUMOR FORMATION

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Fibroblasts from transgenic mice deficient in p16 require only the introduction of activated mutant ras to induce a malignant phenotype characterized by anchorage independent growth (Cell 85:27, 1996).

We have substantiated these findings in a human melanoma model. A low invasive potential melanoma deficient in transcription of p16 message was transfected with activated N-or H-ras. Selected clones of the transfected melanomas showed elevated ras expression and produced a distinctive in vitro phenotype: anchorage independent growth in soft agar, increased migration on tissue culture plastic, and increased invasion through a Matrigel barrier. When injected into SCID mice, the mutant ras transfected clones showed increased local tumor formation, correlating with increased in vitro function. Evidence of distant metastases was not seen in the mutant ras clones studied to date.

Induction of anchorage independent growth involves both the stimulation of proliferation and the resistance to apoptosis (survival) in cells deprived of anchorage-dependent signals. We propose that activating ras mutations directly stimulate three parallel functional pathways that induce local tumor growth in a dermal environment: cell migration and invasion, cell survival, and proliferation.

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P16^{INK4} GENE MUTATIONS IN SQUAMOUS CELL CARCINOMAS OF HUMAN SKIN.

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The p16^{INK4} gene encodes an inhibitory protein of cyclin-dependent kinase 4 (Cdk4) which regulates the cell cycle. Deletions and/ or mutations in the p16 gene have been reported in several kinds of human cancer. However, no information is available about p16 gene mutation in human skin cancers. In this study, mutations in all coding exons (exon 1 to 3) of the p16 gene, including exon 1B, were screened in 21 squamous cell carcinomas (SCCs) of human skin by polymerase chain reaction-single strand conformation polymorphism analysis (PCR-SSCP). Mutations of the p16 gene were detected in 3 of 21 SCCs (14%), which had a mutation of CCC→TC at codon 75 resulting in a frameshift, a nonsense mutation of CGA→TGA at codon 80, and a deletion of 21 base pairs from codon 84 to 90, respectively. The first two mutations are predicted to produce truncated proteins. The last is supposed to inactivate Cdk4 inhibitory function of p16 protein because a mutant p16 protein by a missense mutation in codon 87 has been reported to be defective in interaction with Cdk4. Thus, we concluded that p16 gene mutations played a role of the carcinogenesis in some SCCs of human skin.

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UV INDUCED G2/M PHASE ARREST RESULTS IN DEPHOSPHORYLATION OF THE RETINOBLASTOMA PROTEIN BY PROTEIN PHOSPHATASE TYPE 1.

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The retinoblastoma tumour suppressor gene product (pRB) is a differentially phosphorylated nuclear phosphoprotein which functions in the negative regulation of the cell cycle in G1 by binding to and inactivating the E2F family of transcription factors. Phosphorylation of pRB at the G1/S phase boundary by a CDK4/cyclin D kinase results in release of E2F and subsequent progression through the cell cycle. Hyperphosphorylation occurs at the G2/M phase boundary by cdc2/cyclin B kinase activity and leads to the binding of pRB to the mitotic spindle and microtubule organising centres. Dephosphorylation during late mitosis results in the growth inhibitory, hypophosphorylated form of pRB being segregated to both daughter nuclei after cell division. Okadaic acid (OA) is a potent tumour promoter and functions by inhibiting the activity of protein phosphatase types 1 (PP1) and 2A (PP2A) at micro and nanomolar concentrations respectively. This study has investigated the role of pRB in the G2/M phase block induced by UV-B irradiation of synchronised keratinocytes, which arrest in all phases of the cell cycle. Irradiation of G2/M phase synchronised keratinocytes resulted in the dephosphorylation of pRB and G2/M phase growth arrest. Treatment with low levels of OA (0.5 nM) had no effect on G2/M phase arrest, however, high levels of OA (0.5 μM) abrogated this UV-B induced G2/M phase growth arrest. The results demonstrate a possible function of pRB in UV induced G2/M phase growth arrest due to dephosphorylation of pRB by PP1.

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AN EXAMINATION OF THE MECHANISM BY WHICH GADD153 INHIBITS NF-IL6-MEDIATED TRANSACTIVATION OF THE HUMAN KERATIN 6b GENE PROMOTER. Ian McKay, Elena Volyanik*, Stephen Bustin* and Irene Leigh. Academic Departments of Dermatology and *Surgery, St Bartholomew's and the Royal London Hospital School of Medicine and Dentistry, London, UK.

Keratinocytes in SCCs and psoriasis show aberrant expression of keratin 6 (K6) genes, including K6a and K6b. We have found that NF-IL6 binds to a near-palindromic DNA motif (PAL) in the K6b gene promoter and activates its transcription. As NF-IL6 induces other genes aberrantly expressed in activated keratinocytes it is a potential target for therapeutic intervention.

GADD 153 is known to inhibit NF-IL6 activity and is therefore a model therapeutic. In transient assays of gene expression, using a cytomegalovirus (CMV) promoter/GADD153 gene plasmid co-transfected with CMV/NF-IL6 and K6b gene promoter/CAT reporter plasmids into NIH-3T3 cells, we confirmed that expression of GADD153 reduces NF-IL6-mediated transactivation of the K6b gene promoter in a dose-dependent manner.

To examine the mechanism of this inhibition, we used synthetic double stranded DNA oligos corresponding to the PAL sequence in mobility shift assays with recombinant GADD153 and NF-IL6 proteins. Our findings were, firstly, that NF-IL6 binds to the wild type PAL sequence but not to a synthetic mutant form and, secondly, that GADD153 does not inhibit NF-IL6 binding to the PAL sequence but rather allows formation of alternative complexes at that site. Finally we found that NF-IL6 will not bind to a PAL-equivalent sequence from the K6a gene promoter but that GADD153/NF-IL6 complexes do. This suggests that GADD153 may be a general modulator of K6 gene expression and has implications for therapeutic approaches to SCC and psoriasis.

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DISRUPTION OF ACTIN STRESS FIBERS OF FIBROBLASTS BY CULTURE IN FREE FLOATING COLLAGEN GEL OR CYTOCHALASIN D INDUCES SIMILAR GENE REGULATION BY PARTLY DIFFERENT SIGNALING PATHWAYS. Ch.A. Lambert, B.V. Nusgens and Ch.M. Lapière. Laboratory of Connective Tissues Biology, University of Liège, Belgium.

Two different procedures were used to investigate the intracellular signaling operating in regulation of specific genes that is mediated by mechanical forces and actin stress fibers in skin fibroblasts (F). Culture of F in a retracting free floating collagen gel (CG) or treatment of a F monolayer on a collagen coat by cytochalasin D (CD) disrupts the actin stress fibers and induces a down regulation of collagen $\alpha 1(I)$ while interstitial collagenase (MMP1) is strongly up-regulated. Both regulations need protein synthesis since cycloheximide blocked the effects of CD and CG on the MMP1 and $\alpha 1(I)$ expression. Inhibition of tyrosine kinases also suppressed the CG- and CD-induced regulations of the MMP1 and $\alpha 1(I)$ expression. No or little effect was obtained by blocking receptor tyrosine kinases, phospholipase C, phosphatidylinositol 3-kinase, protein kinases A and G, calmodulin-dependent kinase, cyclooxygenase or by activating the GTP binding protein G_i. Inhibiting the protein kinase C activity by bisindoyl maleimide did not affect the regulation of the two genes induced in CG but suppressed the regulation of the MMP1 induced by CD without modifying $\alpha 1(I)$. These results support a divergent intracellular signaling pathways in the integrin-mediated and cytoskeleton-disrupting drug-induced regulation of MMP1.

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INDUCTION OF HEME OXYGENASE-1 (32-kD HEAT SHOCK PROTEIN) BY A CYTOTOXIC PROSTAGLANDIN, Δ^{12}-PGJ_2 IN TRANSFORMED ENDOTHELIAL AND EPIDERMAL CELLS IN CULTURE. Kouichi Ikai, Hitoshi Kudo, Ken-ichi Toda and Masanori Fukushima*, Department of Dermatology, Kyoto University Graduate School of Medicine, Kyoto and *Department of Internal Medicine, Aichi Cancer Center, Nagoya, Japan

Cyclopentenone prostaglandins (PGs) such as Δ^{12}-PGJ_2 have been demonstrated to inhibit cell proliferation in various cells both *in vitro* and *in vivo*. These PGs are actively transported into cells by a specific carrier on the cell membrane and accumulate in cell nuclei by binding to nuclear proteins. Cyclopentenone PGs induce the expression of several specific proteins, such as 72-kD heat shock protein (HSP72), the expression of which is followed by cell cycle arrest at the G_1 phase at the IC_{50} concentration.

To clarify the mechanism of cytotoxicity of these PGs, the effects of Δ^{12}-PGJ_2 on protein synthesis were examined in transformed mouse endothelial (F2) and epidermal (PAM212) cell lines. Immunoblot analysis of cell lysates using an antibody specific for heme oxygenase-1 (HSP32) revealed that 12 hr incubation with 5 μg/ml Δ^{12}-PGJ_2 induced HSP32 formation in F2 and PAM212 cells. HSP32 was also induced by heat shock treatment at 43°C for 90 min. In contrast, HSP72 was induced in PAM212 cells but not in F2 cells by heat shock and by Δ^{12}-PGJ_2 treatment. The quantity of HSP 32 and HSP 72 produced was markedly decreased by co-treatment with 1 μg/ml of cycloheximide in Δ^{12}-PGJ_2-treated cells. These results suggest that heme oxygenase-1 induced by Δ^{12}-PGJ_2 plays a role in metabolic regulation in epidermal cells as well as endothelial cells.

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IFN-γ-INDUCED IP-10 mRNA EXPRESSION IN CULTURED HUMAN KERATINOCYTES IS UNDER CONTROL OF PROTEIN KINASE-C, TYROSINE KINASE AND cAMP.

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IFN-γ-induced IP-10(γ-IP-10) is a member of the superfamily of chemokines, which are increasingly recognized to play important roles in mediating inflammation and cellular growth. γ-IP-10, present in inflammatory skin diseases and cutaneous T cell lymphoma, has chemotactic and adhesion promoting properties and inhibits angiogenesis. In Raji cells binding of IFN-γ to its receptor leads to phosphorylation of the intracytoplasmic part of the receptor. In fibroblasts a downstream event is the tyrosine phosphorylation of a transcription factor. The signal transduction pathway leading to γ-IP-10 expression in keratinocytes is unknown. The involvement of protein kinase(PK)-C was demonstrated with specific inhibitors (H-7 and Calphostin C) resulting in decreased γ-IP-10 mRNA levels. Moreover depletion of PK-C by pretreatment of the cells with PMA also down-regulated γ-IP-10 mRNA expression. Inhibition with genistein of tyrosine kinase, involved in the transduction of IFN-γ-induced signals, appeared to reduce the γ-IP-10 mRNA expression. Elevated cAMP levels were also shown to reduce γ-IP-10 mRNA expression as could be concluded from experiments with forskolin, W-7 and pentoxyfillin, substances which directly or indirectly raise the cAMP level. These data show that the events leading to the γ-IP-10 mRNA expression in keratinocytes constitute a complex process delicately regulated at different levels in the signal transduction pathway.

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IDENTIFICATION OF A CELL SURFACE MOLECULE ASSOCIATED WITH PROLIFERATION DURING DEVELOPMENT AND CARCINOGENESIS IN HUMAN KERATINOCYTES. **P Kaur, S Paton, J Furze, J Wrin. Hanson Centre for Cancer Research, IMVS, Adelaide, SA, AUSTRALIA.**

We aimed to identify cell surface components which have an important role in human keratinocyte carcinogenesis. We utilized a unique series of human keratinocyte cell lines which mimic specific stages in the progression to squamous cell carcinoma as immunogens to generate monoclonal antibodies (Mabs) to cell surface molecules (CSM) preferentially expressed by tumourigenic keratinocytes compared to normal keratinocytes. One of these Mabs named 10G7, binds to a 130kDa CSM which although undetectable in vivo in normal adult keratinocytes, appears to be induced upon placing these cells in culture. Interestingly, this expression is downregulated in confluent and differentiating keratinocytes. The correlation of 10G7 antigen (ag) expression with proliferation in vitro, could be extended to hyperproliferative conditions in vivo. Firstly, epidermal cells in developing foetal tissues exhibited strong reactivity with Mab 10G7. Secondly, both benign and malignant epithelial tumours of the genital tract and breast expressed 10G7 ag, whereas no reactivity was observed in normal genital and breast epithelium. A functional role for the 10G7 ag in proliferation has been demonstrated by the ability of Mab 10G7 to stimulate proliferation of epithelial cells in culture. Interestingly, other studies indicate that Mab 10G7 can be used to enrich for human epidermal progenitor cells and may permit the isolation of stem cells from the basal cell population. Thus, we have defined an interesting CSM which appears to have a significant role in epidermal proliferation both during development and carcinogenesis.

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BINDING OF TRANSCRIPTION FACTOR SP1 TO THE PROMOTOR REGION OF THE HUMAN BM-40 GENE. Roswitha Nischt, Martin Hafner and Thomas Krieg. Department of Dermatology, University of Cologne, Germany

BM-40 (SPARC, osteonectin) is a highly conserved matrix-associated protein that is found in basement membranes, bones and remodeling tissues. BM-40 expression is spatio-temporally regulated during vertebrate development and in adults suggesting a versatile function of BM-40 in the extracellular matrix.

The promoter region of the human BM-40 gene is characterized by lack of a TATA- and CAAT- box and by the presence of two purine-rich sequences, GGA-box 1 and GGA-box 2. Transient transfection analyses of luciferase reporter gene constructs of different BM-40 promoter fragments revealed that the purine-rich region comprises several distinct regulatory domains. Both boxes contain upregulatory cis-elements. GGA-box 1 is thereby a prerequisite and sufficient by itself for maximal BM-40 transcription.

In electrophoretic mobility shift assays (EMSA) synthetic double stranded oligonucleotides corresponding to GGA-Box 1 and GGA-box 2 are bound by several nuclear factors. One of these factors is binding to both GGA-boxes as shown by crosscompetition. Since sequence analysis revealed several consensus sites for low affinity binding of the transcription factor Sp1 competition with an Sp1 oligonucleotide was performed indicating that Sp1 is the nuclear protein binding to both GGA-boxes. These data were confirmed by super shift EMSA using the Sp1-specific monoclonal antibody Pep 1.

The organization of the human BM-40 promoter might explain the broad BM-40 expression observed in embryos and adults since it displays structural similarities to the promoters of "housekeeping genes" that are characterized by lack of TATA- and CAAT-boxes and the presence of several Sp1 binding sites.

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BM-40 (OSTEONECTIN, SPARC) IS EXPRESSED IN THE EPIDERMIS OF ADULT HUMAN SKIN

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BM-40 (Osteonectin, SPARC) is a calcium binding glycoprotein which has been implicated in the control of cell shape, migration and proliferation. In situ hybridization studies on the expression of BM-40 mRNA in murine tissues have demonstrated the highest levels of transcripts in bone, but the expression was also observed in several other mesenchymal tissues. In contrast, little is known about the expression of BM-40 in adult human skin.

Total RNA obtained from normal human skin was analysed by northern blotting which revealed a marked expression of BM-40. To further localize its expression in the skin, in situ hybridization was performed demonstrating that BM-40 mRNA is expressed in fibroblasts , smooth muscle and endothelial cells in the dermis. Interestingly BM-40 was also detected throughout the basal, spinous and granular layers in the epidermis of adult human skin. These findings were confirmed by immunohistochemistry revealing a marked deposition of BM-40 in the dermis which was most intense directly below the basement membrane in the papillary dermis and around vascular as well as glandular structures. In the epidermis BM-40 could be detected intercellularly in suprabasal cell layers. This finding is further supported by the intercellular deposition of BM40 as detected by immunofluorescence in the keratinocyte line HaCaT. As BM-40 is thought to play a role in processes associated with an invasive cell phenotype its expression was studied in basal cell carcinoma the most common invasive skin tumor. Here the intercellular pattern was pronounced in the outermost layer of the tumor islands accompanied by an increase in staining intensity being observed in the surrounding connective tissue.

This study demonstrates that BM-40 which has previously been thought to be exclusively expressed in extracellular matrix producing cells in fact may play a role in differentiation and maintenance of the epidermis.

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CYCLIN E EXPRESSION IN KERATINOCYTE DISEASES.

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We have recently shown that overexpression of cyclin D protein, one of the G1 cyclins, is seen in nearly half of cases of actinic keratosis (AK) and squamous cell carcinoma(SCC), and suggested a stimulatory role of cyclin D in tumor cell proliferation. Another G1 cyclin, cyclin E has been indicated as a marker of tumor proliferation of breast cancer. We examined the expression of cyclin E by immunohistochemistry in dermatitis, psoriasis, and various keratinocyte tumors to elucidate the role of cyclin E in these conditions. No expression was seen in dermatitis. In psoriasis, keratinocytes in upper epidermis were stained. In premalignant lesions such as AK and Bowen's disease, positive cells were mainly located in suprabasal epidermal layers. In SCC, however, tumor cells were frequently negative for cyclin E. These results suggest that cyclin E is not a good marker for keratinocyte carcinogenesis and cyclin E may have suppressive effect on keratinocyte proliferation rather than stimulatory effect.

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ANALYSIS OF AGE-RELATED VEGF CONTENT IN HEMANGIOMA CHILDHOOD SERA

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Childhood hemangioma constitutes an original feature in the pathology of uncontrolled angiogenesis. Vascular endothelial growth factor (VEGF), a selective mitogen for dermal endothelial cells, may play a role in this pathology where the activation of a paracrine angiogenic factor is suspected. Thus we identified and quantified VEGF from sera of children aged 5 months to 11 years using Western blot analysis and ELISA respectively.

VEGF amounts in sera from children with hemangiomas were twice as high as those in healthy controls and were independent of age (e.g. 142% increase in VEGF in 5 month old children with hemangiomas).

In addition VEGF amounts in sera decreased age-dependently in both hemangioma and normal subjects.

In conclusion, these results indicate an over production of VEGF in younger children and support the hypothesis that VEGF could act as a regulatory factor of angiogenesis in infantile hemangioma.

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EXPRESSION OF NM 23 ("NON METASTATIC") PROTEIN IN KAPOSI`S SARCOMA: AN IMMUNOHISTOCHEMICAL ANALYSIS

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In experimental systems it has been shown that tumor cells of low metastatic potential demonstrated significantly greater nm23 m-RNA levels than related metastatic tumour cells (1). Several studies in different human cancers support the hypothesis that the down-regulation of nm23 gene might be important in metastasis and invasion.

By immunohistochemistry we studied the expression of nm23 protein using the APAAP- technique in 33 cases of Kaposi`s sarcoma (KS). In KS there was a positive reaction for nm23 as a diffuse intracytoplasmatic positive reaction in 15.1% (5/33) of KS. All cases were AIDS-associated KS. The cases showed "angiomatous" and "fibroblastic"- like parts. Nm23 immunostaining was seen in normal epidermis as an internal control.

Considering the hypothetic function of the nm23 gene these results would postulate a high metastatic potential of KS, but we know that the clinical course and the histopathological findings implicate a benign lesion of KS. We thus conclude that the nm23 expression does not seem to be a marker for regulation of metastasize.

1. Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta L, Sobel ME. Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst (1988) 80: 200-204

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OVEREXPRESSION OF THE EGF-RECEPTOR cDNA IN FORESKIN DERIVED KERATINOCYTES CAUSES EPIDERMAL HYPERPLASIA IN RAFT CULTURE.

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Chronic inflammatory skin lesions, e.g. psoriasis, are characterized by acanthosis and aberrant differentiation in addition to leucocytic infiltration. Little is known about the detailed mechanisms leading to epidermal hyperplasia, but dysregulation of several keratinocyte growth factors and their receptors has been described in psoriatic lesions. While the EGF-receptor is only expressed by basal cells in normal skin, expression of the EGF-receptor in all viable layers of psoriatic skin has been observed by immunohistochemistry. Similar changes have been detected for TGF-α, a known ligand and activator of the EGF-receptor. Transgenic mice overexpressing TGF-α develop skin changes similar to psoriasis. Interpretation of these results however is complicated by possible interactions between keratinocytes and infiltrating cells.

To analyse the effect of increased activation of the EGF-receptor pathway on epidermal growth and differentiation, we have transfected human foreskin derived keratinocytes with a retroviral vector expressing the EGF-receptor cDNA. After G418 selection, the keratinocytes were seeded onto collagen embedded fibroblasts and grown as raft cultures at the air-liquid interphase. As expected, the resulting epidermal equivalents exhibited adequate signs of terminal differentiation with expression of suprabasal keratins. Interestingly, raft cultures expressing the EGF-receptor show marked hyperplasia when compared to epidermal equivalents generated from keratinocytes transfected with the retroviral vector alone.

These date strongly support the hypothesis that increased or aberrant activation of the EGF-receptor pathway is sufficient for the development of epidermal hyperplasia and may contribute to similar changes observed in inflammatory skin diseases.

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VASCULAR ENDOTHELIAL GROWTH FACTOR IS CONSTITUTIVELY EXPRESSED IN NORMAL SALIVARY GLANDS AND IS SECRETED IN SALIVA OF HEALTHY INDIVIDUALS.

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Vascular endothelial cell growth factor (VEGF) is a potent mitogen for endothelial cells and is able to induce vascular hyper permeability. It is upregulated during wound healing and has been demonstrated to be overexpressed in a large number of malignant tumors where it is thought to contribute to the formation of tumor vessels and thereby influencing tumor growth and metastasis. When we studied tissue distribution of this factor in organs of adults we found that VEGF mRNA and protein is constitutively expressed in normal salivary gland tissue. Strong VEGF expression as assessed by immunohistochemistry and in-situ-hybridization was detectable mainly in acinar cells. In inflammatory salivary gland lesions, VEGF expression was also found upregulated in ductal elements. In analogy to what we observed in regular acinar cells, acinic cell carcinomas stained strongly for VEGF protein whereas only little was detectable in most other salivary gland tumors. Using an ELISA we found up to 1 ng of VEGF regularly present in saliva of healthy indivi-duals. The strong constitutive expression of VEGF in salivary glands and the secretion of VEGF in saliva suggest an important role for this cytokine in the maintenance of the homeostasis of mucous membranes. Rapid induction of neoangiogenesis by salivary VEGF may help to accelerate wound healing within the oral cavity.

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MITOGENIC EFFECTS ON 3T3 FIBROBLASTS BY BLEOMYCIN-STIMULATED PBMN FACTORS.

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To evaluate the role of bleomycin for tissue fibrosis, effect of bleomycin-derived peripheral blood mononuclear cell (PBMN) factors on in vitro proliferation of mouse 3T3 fibroblasts were investigated. PBMNs were incubated with $10^{-3}\sim10\,\mu g/ml$ of bleomycin, and the culture supernatant (conditioned media; CM) were collected 48h later. Fibroblasts proliferated in a dose-dependent manner by stimulation with CM (1066 ± 124 dpm), but not with control media (608 ± 47 dpm).

Among the populations of PBMN, macrophage showed most intense growth stimulatory activity for fibroblasts. The growth activity was partially inhibited by anti-PDGF (23%), anti-bFGF (20%), anti-IL-1β (15%), anti-TNF- α (10%), and anti-TGF-β (5%) antibodies. Coincubation with CM for 24h induced expressions of IL-1α and IL-6 mRNA on 3T3 fibroblasts. These results suggest that bleomycin stimulates macrophage to release a growth factor-like activity for fibroblasts, which may be due to PDGF and bFGF.

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EFFECT OF FIXATION ON THE AMPLIFICATION OF NUCLEIC ACIDS FROM PARAFFIN-EMBEDDED MATERIAL BY THE POLYMERASE CHAIN REACTION.

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Amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction (PCR) is widely used to detect viral genomes and oncogene mutations in skin specimens. To determine the effect of fixation on the preservation of the nucleic acids, we fixed randomly chosen fresh pathology specimens in Histochoice, formalin and ethanol for 24 and 72 hr and then embedded the tissue in paraffin. Histochoice (Amresco, Solon, Ohio, USA) is a recently developed tissue fixative which contains a mixture of small non-toxic molecules that bind to proteins, carbohydrates and nucleic acids without crosslinking them to their tissue environment. This result in fixed tissue which retain their structure and antigens. Two 5 µm sections were cut from each paraffin block, deparaffinized and then subjected to DNA extraction using standard methods. The effect of fixation was measured by the ability of DNA to serve as a template for the amplification of DNA fragments of about 280 bp in lenght. On amplifying DNA, consistent product was seen in the ethanol and Histochoice specimens after 24 hr of fixation time, whereas variable product was seen with formalin fixation. After 72 hr of fixation, variable results were obtained with Histochoice and ethanol, whereas specimens fixed in formalin were negative. Our results demonstrate the superiority of both ethanol and the non-cross linking fixative Histochoice to that of formalin and the constant deterioration of PCR signal on formalin-fixed material with increased fixation time. The information presented here can be useful for the fixation of current skin specimens, as well as for choosing appropriate archivial material for retrospective studies.

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INCREASED SERUM LEVELS OF INTERLEUKIN-1β AND BASIC FIBROBLAST GROWTH FACTOR IN SEVERE ADAMANTIADES-BEHÇET'S DISEASE.

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To investigate associations between cytokines and Adamantiades-Behçet's disease (ABD), serum levels of interleukin (IL)-1β, IL-8, tumor necrosis factor-α (TNF-α), soluble (s) ICAM-1 and basic fibroblast growth factor (bFGF) were evaluated in 22 patients with ABD (13 male [m], 9 female [f], age 32±10.3 years [y]). Patients were studied during the clinically active stage of the disease (≥1 of symptoms present), 6 of them had severe disease (≥ 2 symptoms). Twelve patients with psoriasis (6 m, 6 f, age 33±9.3 y) and 15 healthy individuals (8 m, 7 f, age 33±9.9 y) served as controls. Serum cytokine levels were measured using ELISA kits.

IL-1β was significantly increased in serum of patients with severe ABD (median [M] 2.3 pg/ml) compared to all patients with ABD (M: 0.31 pg/ml; $p<0.05$) and healthy controls (M: 0.3 pg/ml; $p<0.05$). As there was no significant difference comparing severe ABD to psoriasis (M: 2.3 pg/ml), the increase of IL-1β probably represents a nonspecific inflammatory response. Compared to healthy controls no significant differences in ABD serum levels of TNF-α, IL-8 and sICAM-1 were found.

bFGF was significantly increased in serum of patients with severe ABD (M: 23.9 pg/ml) compared to all ABD patients (M: 3.9 pg/ml), healthy controls (M: 2.2 pg/ml), and psoriasis patients (M: 1.42 pg/ml; $p<0.05$, respectively). It is likely, therefore, that bFGF could be a serum marker which is associated with severe ABD.

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CULTURED ENDOTHELIAL CELLS SECRETE INTERLEUKIN-8 AFTER STIMULATION WITH SERUM OF PATIENTS WITH ADAMANTIADES-BEHÇET'S DISEASE.

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Endothelial cells participate in inflammatory processes by secretion of interleukin-8 (IL-8). To investigate the role of other cytokines on IL-8 secretion by endothelial cells in Adamantiades-Behçet's disease (ABD), immortalized human dermal microvascular endothelial cells (IHDMEC; gift of Dr. Ades, Emory University) were stimulated with serum of patients with active ABD and known levels of IL-1β, tumor necrosis factor-α (TNF-α), and IL-8 as well as with these cytokines alone: IL-1β: 0.1-10 ng/ml, TNF-α: 0.5-50 ng/ml, IL-8: 1-100 ng/ml. After stimulation for 1 h, 4 h and 24 h, IL-8 levels of supernatants were measured using commercial ELISA kits. Compared to non-stimulated IHDMEC (IL-8: 1.5 ng/ml after 1, 4 and 24 h), IHDMEC IL-8-secretion was increased by IL-1β at 10 ng/ml (max. 39 ng/ml after 1 h decreasing to 27 ng/ml after 24 h), by TNF-α at 50 ng/ml (IL-8: 4.5 ng/ml after 1 h and 7.5 ng/ml after 4 h, 24 h) and IL-8 at 100ng/ml (IL-8: 4.5ng/ml after 24 h). Serum of ABD patients only containing raised IL-1β or TNF-α levels as well as these cytokines alone, at concentrations found in patients' serum, did not stimulate IL-8 secretion. In contrast, stimulation of IHDMEC with serum of patients containing several cytokines significantly raised IL-8 levels (4.7-5.2 ng/ml) compared to the cytokines alone (2.6 ng/ml). These results implicate a synergistic interaction of cytokines or an unknown factor and confirm the postulated central role of endothelium in the develpoment of cutaneous lesions in ABD.

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UPREGULATION OF IL-8 RECEPTOR TYPE A AND B-mRNA-EXPRESSION IN TNF STIMULATED HUMAN KERATINOCYTES IS NOT INHIBITED BY ANTI IL-8 ANTIBODIES.

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There is increasing evidence that the expression of C-X-C-chemokines including IL-8 and Gro-α plays an important role in inflammatory skin diseases. TNF-alpha as well as IL-1ß have been reported to be inducers of IL-8 expression in human keratinocytes and recently we showed that IL-8 receptor A and B-mRNA is upregulated in human keratinocytes following induction with TNFα and IL-1β. The extent of induction was dose dependent and the time course was cyclic and similar to own previously reported results on the IL-8 mRNA induction in human keratinocytes. To further investigate the mechanisms of IL-8 receptor mRNA induction we treated cultured human keratinocytes with IL-8 and studied the time course of IL-8 and IL-8 receptor mRNA expression. Using semi-quantitive RT-PCR with intron spanning primers, we observed transient and cyclic increases of IL-8 and IL-8 receptor A and B mRNA transcription during 48 h treatment with IL-8. Interestingly the receptor mRNA concentration always reached its maximum when the IL-8 mRNA was downregulated and vice versa. To evaluate the assumption that the expression of IL-8 and IL-8 receptor may act in concert after stimulation we treated keratinocytes with TNFα and anti IL-8 antibodies and again studied the time course of IL-8 and IL-8 receptor mRNA expression. Unexpectedly the expression of IL-8 receptor mRNA was upregulated indicating that in vitro TNFα increases the expression of IL-8 receptor mRNA in human keratinocytes via mechanisms which are different from the regulation of IL-8 expression.

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GLUCOCORTICOID MODULATION OF CYTOKINE PRODUCTION FROM HAPTEN STIMULATED CULTURED KERATINOCYTES.

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In order to clarify the mechanism of induction of contact dermatitis,we investigated the cytokine producition from chemically-stimulated-cultured keratinocytes.Furthermore,we assessed the modulatory effect of glucocorticoid on the cytokine production from stimulated keratinocytes to confirm the phenomenon that low concentration of glucocorticoid inhibits cytokine production .Various chemical substances such as 2,4,6-trinitrobenzenesulfonic acid sodium salt(TNBS) as sensitizer,methyl salicylate as an irritant,and staphylococcal enterotoxin B as superantigen,were added in culture medium with various concentration of glucocorticoid at the stimulation of cultured keratinocytes. Cytokine contents in culture supernatant were assessed by ELISA.IL-1α but not TNFα or IL-10 was significantly upregulated by sensitizer(TNBS).IL-1α in TNBS stimulated keratinocytes was upregulated by low concentration(10^{-10}M) of hydrocortisone nine times more than normal condition.These result suggests that glucocorticoid might modulate cytokine production of hapten stimulated keratinocytes through glucocorticoid receptor.

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PRODUCTION AND REGULATION BY INTERLEUKIN-1 OF THE NEUTROPHIL CHEMOATTRACTANT gro-α IN MURINE KERATINOCYTES.
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Neutrophil accumulation is a prominent part of the response to IL-1 in both human and murine skin, and although IL-8 is produced by keratinocytes in response to IL-1 in man, IL-1 inducible neutrophil chemoattractants remain to be identified in murine keratinocytes. To explore this issue further, we have sought the presence of the potent neutrophil attractant chemokine gro-α in murine keratinocytes and examined its regulation by IL-1 both in vitro and in vivo.

By northern blot analysis under resting conditions, mRNA encoding gro-α was undetectable in the murine keratinocyte line PAM212, but following stimulation with IL-1, this message was rapidly induced. Similarly, no gro-α mRNA could be detected in normal murine epidermis *in vivo*, but following topical application of PMA (which results in a neutrophilic inflammatory response), gro-α mRNA was significantly induced. PMA induced marked overinduction of this message in transgenic mice that overexpress type-1 IL-1 receptor (IL-1R1) in basal keratinocytes, indicating that this signal was dependent upon IL-1 mediated keratinocyte activation. Furthermore, transgenic mice that overexpress IL-1α in keratinocytes constitutively expressed low levels of gro-αmRNA, and double transgenic mice that overexpressed both IL-1 and IL-1R1 (and develop a spontaneous cutaneous inflammatory cell infiltrate including neutrophils) expressed epidermal gro-α mRNA at very high level.

These data demonstrate that murine keratinocytes inducibly express the potent neutrophil attractant chemokine gro-α, and that IL-1 can play a major role in regulation of its expression. We hypothesize that gro-α may play an analogous role in murine skin to that played by IL-8 in man, and represent an important keratinocyte-derived regulator of inflammatory responses in the skin.

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SELECTIVE INHIBITION OF TISSUE DESTRUCTION BY THERAPEUTIC IMMUNE DEVIATION OF ESTABLISHED CONTACT HYPERSENSITIVITY (CS).
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Delayed type hypersensitivity reactions (DTHR), mediated by Interferon-γ producing and IL-4 deficient CD4+ T helper cells (Th1) are protective when directed against intracellular pathogens or tumors. However, they may become harmful if directed against haptens or self epitopes and mediate autoimmune diseases such as psoriasis, lichen planus, CS or graft rejection. In contrast, IFN-γ deficient, IL-4 producing Th2 do not mediate but may even prevent DTHR. Since IL-4 is capable of preventing Th1 development and to deviate activated T cells towards a Th2 phenotype, we investigated the role IL-4 induced immune deviation in the therapy of established CS, an ongoing DTHR. Mice were sensitized with TNCB and challenged on day 5 either in the presence or the absence of IL-4 (0.3 mg/day). As described by others, IL-4 application reduced ear swelling. More importantly, when rechallenged 5 days later with TNCB only, in IL-4 treated animals ear swelling was reduced by up to 70%. To investigate, whether immune deviation might still be effective after repetitive hapten exposure, CS was elicited and all mice were challenged on day 5. Seven days later, mice were challenged a 2nd time and one half received IL-4. At the 3rd challenge 5 days later (day 22) with TNCB alone, ear swelling was reduced by 40-70% in the IL-4 treated animals. Most importantly, H&E stained sections revealed that IL-4 treated animals had a normal lymphocytic infiltrate in the dermis, but almost no neutrophils and no tissue destruction within the epidermis. Since immune deviation did not reduce hapten-specific IL-2 production but a sustained IL-4 production by CD8+ T cells, immune deviation did not prevent from inflammation but protected against proinflammatory tissue destruction. Therapeutic immune deviation may become a novel therapeutic strategy for Th1-mediated autoimmune diseases such as psoriasis or CS.

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THE ROLE OF TNFα AND LTα IN B CELL PROLIFERATION AND IgE SYNTHESIS.
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The cytokines tumor necrosis factor alpha (TNFα) and lymphotoxin alpha (LTα) are produced many different cells including B lymphocytes. We could recently show that stimulation of human B cells by anti-CD40 mAb alone and in combination with IL-4 results in TNFα and LTα production. The aim of this study was to evaluate the role of these cytokines in B cell proliferation and IgE synthesis. PBMC and B cells from normal healthy donors were cultured in the presence of TNFα and LTα alone and in combination with anti-CD40 (1μg/ml) + IL-4 (5ng/ml). Proliferation of cells was measured by [H^3] incorporation and IgE was detected in the supernatants after 10 days of cell culture by ELISA. Our results show that TNFα and LTα (1-1000 pg/ml) stimulated 2-3 fold B cell proliferation in a dose dependent manner. Furthermore addition of either TNFα or LTα to anti-CD40+ IL-4 stimulated B cells enhanced anti-CD40+ IL-4 induced proliferation (20-40%). By using neutralizing mAb against TNFα and LTα (0.01-1μg/ml) in anti-CD40+IL-4 stimulated B cells a modest inhibition of B cell proliferation was detected (anti-TNFα 25.5% and anti-LTα 43% inhibition, n=3). TNFα and LTα induced on the other hand no IgE synthesis in B cell cultures by themselves. Addition of neutralizing mAb against TNFα and LTα (0.01-1μg/ml) to anti-CD40+IL-4 stimulated B cells resulted however in a definite partial inhibition of IgE synthesis. In conclusion the data indicates that TNFα and LTα are involved in CD40+IL-4 stimulated proliferation and IgE synthesis of human B cells. This activity can be further amplified by addition of either cytokines to the stimulated cells. Since TNFα and LTα are upregulated in a variety of cutaneous inflammatory diseases the in vitro results may explain the clinically observed increased IgE levels in these patients.

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INVOLVEMENT OF INTERLEUKIN-5 IN DRUG-RELATED HYPERSENSITIVITY SYNDROME WITH EOSINOPHILIA.
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The denomination of "hypersensitivity syndrome" (HSS) became widely used to describe severe drug rashes associated with systemic symptoms and blood eosinophilia. Interleukin-5 (IL-5) is considered the most important cytokine involved in the regulation of eosinophilia. Increased levels of blood IL-5 were found in patients with eosinophilia either secondary to parasitic infections or idiopathic. The aim of our study was to determine if IL-5 was also implicated in drug-related eosinophilia in patients with "hypersensitivity syndrome".

Plasma levels of IL-5 were determined by ELISA (Quantikine®, R&D systems, Abingdon, UK) in 6 patients with HSS and eosinophilia above 1,500/mL and in 7 patients with drug eruptions not associated with eosinophilia. IL-5 levels were normal in all 7 cases of drug eruptions without eosinophilia and elevated in 3 cases of HSS. In patients with HSS sequential measurements showed that IL-5 production peaked several days before the maximum eosinophilia and returned to normal in a few days even when eosinophilia persisted. All 3 patients with HSS and normal IL5 levels had blood samples taken after the peak of eosinophilia.

As expected our results show that IL-5 is involved in eosinophilia of drug-related HSS. IL-5 production seems restricted to the early stages of the reaction. That kinetic suggests that plasma IL-5 was mainly produced by activated lymphocytes rather than by eosinophils. Further in vitro studies of IL-5 production by patients lymphocytes stimulated by the suspect drugs, and/or metabolites, could provide a usefull tool in establishing the causality of HSS with eosinophilia.

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TOPICAL FK506: SUPPRESSION OF TH_1 AND TH_2 CYTOKINE INDUCTION IN LYMPH NODE CELLS IN VIVO.
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Recently, therapeutical efficacy of FK506 has been shown in inflammatory skin diseases, such as atopic dermatitis and psoriasis. The aim of this study was to investigate immunosuppressive effects of topical FK506 on cytokine expression in lymph node cells in vivo. On four consecutive days NMRI mice were topically treated on the dorsal surfaces of both ears with increasing concentrations of FK506 (0.01- 1%). During the last three days, mice received an additional topical treatment with the contact sensitizer oxazolone (1%). On day 5, local draining lymph nodes of the ears were removed, lymph node cell counts were assessed and total mRNA was extracted. After reverse transcription, a competitive polymerase chain reaction with primers for β-actin, IFN-γ, IL-2 and IL-4 was performed. Cytokine mRNA levels for IFN-γ, IL-2 and IL-4 were increased during the induction phase of contact hypersensitivity in lymph node cells, compared to the vehicle control. Topical treatment with 0.01-1% FK506 dose-dependently suppressed both Th_1 cytokine (IFN-γ, IL-2) and Th_2 cytokine (IL-4) mRNA expression in lymph node cells. Flowcytometric analysis of intracellular cytokine production confirmed the results obtained at the mRNA level. These results give insights into the mechanisms of action of topical FK506 in the treatment of inflammatory skin diseases and suggest that topical FK506 may act by modulation of Th_1/Th_2 dysbalance in different stages of atopic dermatitis.

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OXIDATION IS INVOLVED IN KERATINOCYTE-DERIVED TNF-α PRODUCTION
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The skin constitutes an important target organ for oxidation. Oxidation results in tissue damage, inflammation and long-term metabolic changes due to DNA damage such as cancer. We studied the involvement of oxidation in the induction of TNF-α in nontransformed human keratinocytes and SV40-transformed human keratinocyte cell lines. Keratinocytes were cultured to subconfluence and stimulated with phorbol myristate acetate (PMA), interleukin (IL)-1β and UVB light. All agents increased TNF-α at the protein level as assessed by a bioassay and at the mRNA level using RNase protection assay. N-acetyl-L-cysteine (NAC), a reducing agent and precursor for glutathione, inhibited TNF-α production induced by PMA or IL-1β in a dose-dependent manner. This was observed both at the mRNA and protein level. However, NAC did not inhibit UVB-induced TNF-α induction , showing that UVB light does not utilize the same mechanism for TNF-α induction as do PMA and IL-1β. Furthermore, PMA-induced IL-1α and IL-1β production by keratinocytes were not affected by NAC. TNF-α production induced by PMA was further inhibited by PDTC, diamide, N-ethylmaleimide and BCNU showing that TNF-α induction depends on a delicate balance of the intracellular milieu. The inhibitory effect of NAC on PMA-induced TNF-α production could be partially reversed by buthionine sulfoximine, an inhibitor of glutathione biosynthesis. Finally, PMA-induced TNF-α expression could be inhibited by rotenone, an inhibitor of the mitochondrial respiratory chain. These results show that oxidation, possibly of endogenous thiol groups, is involved in the signaling of PMA and IL-1β-induced production of TNF-α. Our results also offer an explanation why NAC inhibits contact dermatitis induced by hapten application to the skin as IL-1β-induced TNF-α production has been shown to be critical in this reaction.

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DOWN-REGULATION OF PRO-INFLAMMATORY TUMOUR NECROSIS FACTOR-α, INTERLEUKIN-6 AND INTERLEUKIN-1β BY *MALASSEZIA FURFUR*

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Malassezia furfur is a dimorphic yeast forming an integral part of the human cutaneous microflora and is implicated in pityriasis versicolor, seborrhoeic dermatitis, *Malassezia* folliculitis and more recently, systemic diseases in immunocompromised individuals. The purpose of this study was to investigate the *in-vitro* capacity of *M. furfur* serovars A, B & C (exponential & stationary phase) to modulate the release of the pro-inflammatory cytokines (TNF-α, IL-6 & IL-1) by human peripheral blood mononuclear cells (PBMNC) (2 healthy donors). Co-incubation (PBMNC together with *Malassezia furfur*) supernatants (up to 48h), together with LPS (+ve) and culture medium (-ve) controls, were assayed by ELISA (IL-6) and bioassay (IL-6, IL-1β, TNF-α). PBMNC viability was determined by MTT cleavage. Maximal cytokine levels were detected at 24h. Levels of TNF-α, IL-6 and IL-1β derived from both PBMNC donors, were significantly reduced ($P<0.05$) when compared with constitutive negative control values. Specific activities for TNF-α were as follows: PBMNC Donor II (serovar B); culture medium 3.5, *M. furfur* 0.63 (MSD = 0.5); PBMNC Donor II (serovar C); culture medium 3.0, *M. furfur* 0.72 (MSD = 1.3). This was invariably found with ratios of 10 *Malassezia*:1 PBMNC & 20 *Malassezia*:1 PBMNC. In addition, stationary phase cells appeared to be more 'immunosuppressive' than exponential cells. These results directly complement previous data revealing significant reductions in PBMNC IL-1β release in the presence of *M. furfur* (Walters *et al*, 1995, Infect Immun **63**, 1223). This data indicates that *M. furfur* modulates the pro-inflammatory response of PBMNC *in-vitro*. This may be important in the pathogenesis of *Malassezia*-associated dermatoses which at worst are, mildly inflammatory.

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INTERLEUKIN-10 IS PRODUCED BY HUMAN KERATINOCYTES THROUGH IgE RECEPTOR (FcεRII/CD23) STIMULATION.

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The synthesis of IL-10 by human keratinocytes has been much controversial. UVA1 and UVB irradiation seems until now to be the only way to induce IL-10 production by human keratinocytes. CD23 is the affinity receptor for IgE (FcεRII/CD23) and we have previously shown that it was inducible on human keratinocytes upon IL-4 stimulation. CD23 ligation leads to the transcription of the inducible Nitric-Oxide Synthase gene (iNOS) and to the release of proinflammatory mediators (TNF-α, IL-6). Recent data suggested that CD23-stimulated human monocytes produced IL-10. We then investigated IL-10 synthesis upon CD23 stimulation on normal human keratinocytes. Keratinocytes were first preincubated with IL-4 for 48 h and CD23 expression was checked using immunostaining. CD23+ keratinocytes were then stimulated with an anti-CD23 mAb or with IgE/antiIgE immune complexes. After 48 h and 72 h, IL-10 levels peaked at 120 pg/ml in culture supernatants. IL-10 mRNA was detectable after 18h of stimulation of CD23+ keratinocytes, both with RT-PCR and blot after PCR products transfer to a nylon membrane. In addition, neutralization of Il-10 with an anti-IL-10 mAb increased both in magnitude and duration TNF-α production. Taken together, these data indicate that that the engagement of the CD23 molecule at the cell surface of human keratinocytes induces the generation of the immunosuppressive cytokine IL-10, and that this generation regulates the production of the proinflammatory cytokine TNF-α, suggesting the presence of a regulatory cytokine network during skin inflammatory diseases.

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INSECT VENOM IMMUNOTHERAPY INDUCES IL-10 PRODUCTION TOGETHER WITH A T_{H2} TO T_{H1} SHIFT IN VENOM ALLERGIC SUBJECTS.

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The current study was carried out to elucidate the immuno-regulatory changes induced by specific immunotherapy (SIT) in bee or wasp allergic patients. All subjects included in this study had a history of severe systemic allergic reactions to stings of the respective insect as well as positive skin tests and RAST with the respective venom. Parameters assessed in peripheral blood mononuclear cells before and after initiation of SIT (rush therapy reaching a maintenance dose of 100 µg venom injected subcutaneously within one week) were: surface marker expression (CD3, CD4, CD8, CD45RA, CD45RO, IL-2Rα, IL-4R, IL-12R, $Fc_\varepsilon RII$, CD40, CD40L) and cytokine producing cells (IFN-γ after stimulation with PMA/ionomycin/monensin) measured by flow cytometry as well as cytokine secretion (IFN-γ, IL-4) measured by ELISA (IL-12 by capture bioassay, IL-10 by PCR) and proliferation after stimulation with the respective venom. Significant decreases were observed after SIT for: proliferative response to venom and venom plus IL-4, IL-4 secretion, $Fc_\varepsilon RII$ as well as CD40 and CD40L expression (the latter being important for isotype switching). Significant increases were observed after SIT for: IFN-γ concering the amount secreted and the number of producing cells (especially among CD8- and CD45RO-positive cells) and IL-10. Addition of blocking anti-IL-10 antibodies but not isotype control antibodies prevented down-regulation of proliferation and enhanced IFN-γ (but not IL-4) secretion. These data indicate that in insect venom allergic subjects SIT not only induces a rapid shift in cytokine expression from T_{H2}- (IL-4) to T_{H1}-type (IFN-γ) cytokines but also leads to induction of the immuno-suppressive cytokine IL-10, which can be produced by T_{H1} and T_{H2} cells in humans and may be important for the limitation of potentially harmful allergen-specific T_{H1} responses. The described changes in cytokine expression may be responsible for subsequent increases in allergen-specific IgG and decreases in IgE production as well as suppressive activity observed in earlier studies.

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DETECTION OF IL-10 RECEPTOR ON HUMAN EPIDERMAL CELLS

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The antiinflammatory cytokine IL-10 was originally described in TH2 cells and characterised by its ability to inhibit the typical response of TH1 lymphocytes. As it has been previously demonstrated that keratinocytes are able to produce IL-10, we were interested whether these cells may also be the targets of these cytokine. Since the effects of IL-10 are mediated through specific receptors, we have studied the keratinocyte IL-10 receptor characteristics. Freshly separated human epidermal cells prepared from the skin of healthy individuals and the spontaneously transformed epidermal HaCat cell line were used for the investigations. For receptor studies the cells were analysed by flow cytometric using biotinylated IL-10 as ligand and avidin-coupled FITC for detection (IL-10/FITC). Incubation of freshly separated human keratinocytes and HaCat cells with increasing concentrations of IL-10/FITC revealed increasing ligand binding to the cells. Addition of unlabelled ligand inhibited the binding suggesting that the IL-10/FITC binding was specific. Furthermore we analysed the expression of the activity of the IL-10 receptor gene using RT-PCR technique, by means we could identify the IL-10 receptor mRNA. Taken together, our results clearly demonstrate the presence of specific IL-10 receptors on keratinocytes, suggesting that human epidermal cells are able to respond to IL-10 signals from other cells which might contribute to the pathogenesis of inflammatory skin diseases.

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EOSINOPHILIC GRANULOCYTES PRODUCE BIOLOGICALLY ACTIVE IL-12

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Eosinophilic granulocytes are thought to play an important role in the pathogenesis of atopic eczema. The initiation phase of atopic eczema is characterized by a switch from a Th_2-like to a Th_1-like in-situ cytokine expression pattern. A hallmark of this early stage of disease is the immigration of eosinophils. We therefore assessed whether eosinophils may be able to synthesize the cytokine IL-12, which is well known for its capacity to promote the differentiation of Th_1-like T-cells. Blood-derived, highly purified (> 95%) human eosinophils were treated in culture with IL-4, IL-5, GM-CSF, IFNγ, TNFα, IL-1α, RANTES, PAF and C5a, respectively. Expression of IL-12 protein (ELISA) and of the mRNAs for the p35 and p40 subunit (semiquantitative RT-PCR) was found to be strongly induced by the Th_2-like cytokines IL-4, IL-5, and GM-CSF, but not by the Th_1-like cytokine IFNγ. TNFα and IL-1α moderately induced both mRNAs and RANTES exclusively induced the p40 subunit. Eosinophil derived IL-12 was biologically active, since supernatants derived from IL-4, IL-5 and GM-CSF treated eosinophils were able to superinduce concanavalin A-induced expression of IFNγ by human Th_1-like T-cells in culture. This effect could be inhibited by addition of neutralizing IL-12 antibodies. In conclusion, eosinophils were found to secrete biologically active IL-12 after treatment with selected biological response modifiers, which mainly represent cytokines of the Th_2-like type. Thus, eosinophils may play a key-role in promoting the switch from the Th_2-like to the Th_1-like immune response in atopic eczema.

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IL-13 SELECTIVELY INDUCES MCP-1 SYNTHESIS AND SECRETION BY HUMAN ENDOTHELIAL CELLS.

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Chemokines secreted by endothelium have been demonstrated to promote leukocyte recruitment to sites of inflammation. We investigated the effect of the T lymphocyte-secreted cytokine IL-13 on endothelial expression of chemokines. Employing *in situ*-hybridization and ELISA techniques we demonstrated that IL-13 which shares many of its activities with IL-4 selectively induces expression of the C-C chemokine monocyte chemoattractant protein-1 (MCP-1) in human umbilical vein endothelial cells (HUVEC). However, it failed to upregulate other C-C and C-X-C chemokines potentially inducible in endothelium as RANTES (regulated on activation normal T expressed and secreted), groα or IL-8. IL-13 dose-dependently induced monocyte chemotactic activity by HUVEC which could be efficiently blocked by neutralizing antisera against MCP-1. In contrast to the synergistic effect of IL-13 and TNF-α on endothelial VCAM-1 surface expression TNF-α-induced secretion of MCP-1 was not augmented by IL-13. Studying the signaling pathway activated by IL-13 it could be demonstrated that a neutralizing mAb to the 140 kDa component of the IL-4R (IL-4Rα) inhibited the effect of IL-13. Immunoprecipitation studies revealed that the endothelial IL-4Rα is rapidly tyrosine-phosphorylated upon treatment with IL-13 and IL-4 which might refer to a redundant action of these two cytokines on endothelial MCP-1 and VCAM-1 expression. Our data are consistent with the hypothesis that IL-13 facilitates recruitment of monocytes and lymphocytes via endothelial expression of MCP-1 and VCAM-1.

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DIFFERENTIAL RELEASE OF IL-4 AND IL-13 FROM IMMUNOLOGICALLY ACTIVATED HUMAN BASOPHILS.

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IL-4 and IL-13 are pleiotropic cytokines which are known to induce IgE secretion from B-cells and thereby play a crucial role in the perpetuation of immediate hypersensitivity responses. Here we report that basophils release both IL-4 and IL-13 at different rates following immunologic activation. Human basophils were purified to 70-90% homogeneity by Ficoll density centrifugation, followed by elutriation and negative selection using Dynabeads. The cells were then incubated for a range of time points (5 min -24 hours) in the presence or absence of anti-IgE and the release of histamine, IL-4 and IL-13 was determined. A rapid release of IL-4 was observed within 10 min which levelled at 30 min and was then followed by a further successive rise in IL-4 reaching maximum levels within 4 hours (652.2 ± 265.0 pg/10^6 basophils, n=5). Lysed, unstimulated basophils were found to contain IL-4 in 70% of donors studied (43.5 ± 8.7 pg/10^6 basophils) indicating that the early release of IL-4 at 5-10 min was from these stores. In contrast, the release of IL-13 was more gradual and no preformed component was observed. IL-13 was detected only after 1 hour incubation and the levels rose to reach a maximum stimulated release between 16 and 24 hours (686.1 ± 224.0 pg/10^6 basophils). By rapidly releasing IL-4 as well as releasing IL-13 over prolonged time periods, human basophils display the capacity to act as orchestrators of IgE-dependent hypersensitivity responses.

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THE EXPRESSION OF IL-15 AND ITS RESPECTIVE RECEPTOR IS ENHANCED BY INFLAMMATORY MEDIATORS.

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Recently we were able to show that the cytokine IL-15 is expressed in human skin and identified keratinocytes as the main source of IL-15 in human epidermis. Furthermore we revealed enhanced expression of this cytokine after application of contact allergens (DNFB, TNCB). After these results we were interested in the question wether the expression of IL-15 is also influenced by other inflammatory mediators like IFN-γ and LPS. Therefore we performed time kinetic experiments of IFN-γ and LPS application and analysed the expression on mRNA level by RT-PCR analysis and in parallel on protein level by FACScan analysis. Our data show an upregulated IL-15 expression after IFN-γ stimulation. As IL-15 binding and signal transduction is mediated via a heterotrimeric complex consisting of the IL-2Rβ and IL-2Rγ chain and a recently cloned IL-15 receptor α chain we were also interested in the expression and regulation of these receptor components after stimulation of human keratinocytes. We obtained an increased expression of the IL-2Rβ and IL-2Rγ chain after IFN-γ stimulation. As IL-15 expression is enhanced by several inflammatory stimuli in human epidermis this cytokine described as a T cell mitogen and chemoattractant has the potency to be an important inflammorty mediator in human epidermis. As our data indicate a coordinated upregulation of IL-15 expression and its receptor we speculate that keratinocytes may respond to IL-15 in an autocrine fashion.

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ISOLATION AND CHARACTERIZATION OF A HUMAN EOTAXIN PRODUCED BY DERMAL FIBROBLASTS

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Accumulation of eosinophils (Eos) in the dermis is a characteristic feature of a number of allergic skin diseases and atopic dermatitis. It has been speculated that Eos are attracted by cell-selective chemotaxins in vivo. Therefore we were interested to investigate, whether dermal fibroblasts represent a cellular source of Eo-selective attractants. We stimulated cultivated dermal fibroblasts with TNFα for various time periods and analyzed supernatants for the presence of Eo attractants by the use of the Boyden chamber technique. Biologically active fractions were purified by heparin-affinity chromatography followed by HPLC separation and were finally analyzed by amino acid sequencing. As a result we detected after 24 hrs stimulation exclusively the chemokine RANTES as Eo-attractant. After 48 hrs stimulation a second heparin-binding Eo-attractant could be identified, which peaked three to four days after starting of stimulation. Purification to homogeneity revealed a 13 kD Eo-selective chemoattractant protein, which was found to be N-terminally blocked. Peptide mapping experiments and subsequent sequencing of the fragments showed very high similarity, but not a complete identity with the deduced sequence of the recently cloned human eotaxin. In addition, SDS-PAGE analyses revealed non-identity with recombinant eotaxin (M_r:10 kD). Thus dermal fibroblasts represent the first natural source of a biologically active human eotaxin. Further biochemical analyses are necessary to investigate whether the differences between natural and recombinant eotaxin come from a post-translational modification or whether the fibroblast-derived eotaxin represents the gene product of a closely related gene.

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HUMAN EOTAXIN ACTIVATES THE RESPIRATORY BURST OF HUMAN EOSINOPHILS.

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Eosinophils are predominant effector cells in allergic diseases such as asthma and atopic dermatitis. They are activated by distinct cytokines and chemokines leading to the immigration in the inflamed tissue and mediate tissue damage by releasing of reactive oxygen species. Herein, the effect of the recently cloned CC chemokine, human eotaxin, was investigated for the ability to affect different eosinophil effector functions and compared to the CC chemokines MCP-3 and RANTES. Human eotaxin induced chemotaxis of human eosinophils in a dose-dependent manner. The range of efficacy of the CC chemokines compared to the well known chemotaxin C5a was eotaxin = RANTES > MCP-3 > C5a. In addition, eotaxin induced rapid and transient actin polymerization, a prerequisite for cell migration, in eosinophils in the same range of efficacy as observed for chemotaxis. To investigate whether eotaxin was able to activate the respiratory burst of eosinophils, release of reactive oxygen species was measured by lucigenin-dependent chemiluminescence (CL). Eotaxin induced production of significantly high amounts of reactive oxygen species at a concentration between 10 ng/ml and 500 ng/ml. Surprisingly, eotaxin was as effective as the most potent eosinophil activator C5a. The range of efficacy of the CC chemokines compared to C5a in the activation of the respiratory burst was eotaxin = C5a > MCP-3 > RANTES. In summary, this study underlines the importance of eotaxin as the most potent activator of the respiratory burst, actin polymerization and chemotaxis. Therefore, eotaxin plays an important role not only by attracting eosinophils to the site of inflammation but also by tissue damaging by its capacity to induce the release of reactive oxygen species.

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HUMAN DERMAL FIBROBLASTS EXPRESS EOTAXIN: MOLECULAR CLONING, AND mRNA-EXPRESSION.

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Recently we discovered and purified a novel ß-chemokine with eosinophil specific chemotactic activity from supernatants of long-term TNF-α stimulated dermal fibroblasts. To further characterise this new peptide and to study its gene expression we used a molecular biology approach:

Based on partial protein sequence data obtained from sequencing peptide fragments of the novel chemokine we designed degenerated specific oligonucleotides and applied these for rapid amplification of specific cDNA ends (RACE). Cloning of the PCR products initially revealed MCP-3 and MCP-1 cDNA fragments indicating that the dermal fibroblasts under study express MCP-3 and MCP-1. After improving the annealing conditions for 5'- and 3'-RACE reactions we were able to clone several novel ß-chemokine cDNA species. Two nearly identical clones share high sequence homology with the peptide fragments obtained from the new chemokine as well as with human MCP-1, MCP-2, MCP-3 and with rodent eotaxins. While there was no matching cDNA sequence accessible in the GenBank/EMBL sequence databases at the time of our sequence submission, recent publications show that one of this cDNA clones is identical with human eotaxin cDNA.

Preliminary semi-quantitative RT-PCR experiments with primers designed to be specific for human eotaxin cDNA indicate constitutive expression of eotaxin mRNA in human dermal fibroblasts which is slightly upregulated by TNF-α. Further studies need to address the question, why eosinophil specific chemotactic activity is detected in fibroblast supernatants only after prolonged stimulation with TNF-α while eotaxin mRNA is constitutively expressed.

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RECOMBINANT HUMAN EOTAXIN INDUCES OXYGEN RADICAL PRODUCTION, CA^{2+}-MOBILIZATION, ACTIN REORGANIZATION AND CD11b UPREGULATION IN HUMAN EOSINOPHILS.

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Eosinophils are major effector cells in the propagation of inflammation in allergic late phase reactions, particularly, atopic dermatitis. Accumulation of eosinophils is presumably caused by complement fragments and chemokins. Recently the novel CXC-chemokine Eotaxin was identified as selective chemotaxin for eosinophils. Here, the biological activities and the activation profile of Eotaxin was further characterized and compared to other eosinophil chemotaxins such as complement fragment C5a (C5a) and RANTES in human eosinophils. Eotaxin stimulated the production of reactive oxygen metabolites as demonstrated by lucigenin-dependent chemiluminescence and superoxide dismutase-inhibitable cytochrome C reduction. Furthermore, Eotaxin induced up-regulation of the integrin CD11b. In addition, fluorescence measurements with Fura-2-labeled eosinophils in the presence of EGTA indicated Ca^{2+}-mobilization from intracellular stores by Eotaxin. Flow cytometric studies revealed rapid and transient actin polymerization upon stimulation with Eotaxin. At optimal concentrations the changes induced by Eotaxin were comparable to those obtained by C5a and RANTES. Cell responses elicited by Eotaxin were inhibited by pertussis toxin indicating coupling of its putative receptor to G-proteins. These results indicate that Eotaxin is a strong activator of eosinophils with comparable biological activity as the eosinophil chemotaxins C5a and RANTES. These findings point to a role of Eotaxin in the pathogenesis of eosinophilic inflammation as chemotaxin as well as activator of pro-inflammatory effector functions.

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MONOKINE INDUCED BY γ-INTERFERON (MIG): A NEW MEMBER OF THE CHEMOKINE FAMILY OF CYTOKINES EXPRESSED IN CULTURED HUMAN KERATINOCYTES AND CUTANEOUS T-CELL LYMPHOMAS.

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MIG is a recently described C-X-C chemokine lacking the ELR motif and is closely related in sequence to γIP-10. To date, little is known about its biological activitites, except for the notion that it is chemotactic for tumor infiltrating lymphocytes and might have angiostatic properties. In contrast, γIP-10 is well studied and is considered to play an important role in skin inflammation and cutaneous T-cell lymphoma (CTCL) due to its chemotactic and adhesion promoting properties.

In order to delineate possible roles of MIG in skin associated diseases, we started a set of experiments to study MIG expression in human cultured keratinocytes (KC). MIG encoding mRNA could only be detected in normal cultured KC by a highly sensitive RT-PCR method, but not by Northern blotting. Upon stimulation of KC with IFN-γ, MIG mRNA expression was increased in a time and dose dependent manner and could be detected by Northern blotting. These observations are similar to γIP-10 mRNA expression; however, in contrast to γIP-10 mRNA, no additional induction of MIG mRNA was found with the combination of IFN-γ and TNFα or IL-4. This time and dose dependent MIG mRNA expression is apparently not regulated via de novo protein synthesis since the inclusion of cycloheximide did not alter these expression levels.

Next we examined MIG mRNA expression by RT-PCR in CTCL. Prelimenary studies on different types of CTCL demonstrated that MIG mRNA, like IP-10 mRNA, is expressed in biopsies taken from patients with mycosis fungoides and lymphomatoid papulosis, but not or rarely in primary cutaneous CD30+ or CD30- large T-cell lymphomas. These results suggest that MIG might play a role in the generation of an anti-tumor response observed in these CTCL.

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TISSUE SPECIFIC EXPRESSION AND ALTERNATIVE SPLICING OF LST1, A NEW GENE IN THE TNF REGION

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The human tumor necrosis factor (TNF) gene cluster on the short arm of chromosome 6 encodes the known cytokines TNFα, lymphotoxin α (LTα)/TNFβ and LT-β , which play an important role in various inflammatory reactions of the skin. In close vicinity to LTB we recently identified a new gene, leucocyte specific transcript 1(LST1). Here we describe the genomic structure of LST1, its complete full-length transcripts, and tissue specific alternative splicing.

Using a modified RACE technique to ensure complete reverse transcription of 5' ends we isolated five different alternatively spliced cDNA clones. By comparison with the genomic sequence seven exons and four introns spanning a total of 2.7 kb were identified. According to Northern blot analysis LST1 is predominantly expressed in monocytes but at low levels also in T cells. In monocytes LST1 transcription is inducible upon stimulation with IFN-γ. Alternative splicing could lead to four different protein isoforms (97 aa, 66 aa, 54 aa, and 28 aa). The 97 aa polypeptide contains a hydrophobic amino terminus that presumably acts as a membrane-anchoring domain whereas the carboxy terminus is homologous to the 54 aa polypeptide which potentially represents a soluble form. In monocytes, IFN-γ induces transcription of the cDNA encoding the 54 aa putatively soluble form; in contrast, in T cells stimulation leads to the preferential transcription of the 97 aa transmembrane isoform. These data suggest that LST1 may have a functional role in signal transduction of monocytes and possibly also T cells, which could be relevant for cell-mediated immunmodulatory effects, or furthermore, serve as a soluble signal transducer in the cutaneous system.

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DNA REPAIR DEFICIENCY IN GENODERMATOSIS : IN VITRO AND IN VIVO ASSAY.

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DNA repair is responsible for removing damage caused by exposure to ultraviolet radiation. *Xeroderma pigmentosum* (XP) is a rare autosomal recessive disease with multiple sunlight-induced skin cancers. XP patient cell studies show DNA repair synthesis defects in response to UV light. Others genodermatosis such as the syndrome of Bazex, Dupré and Christol, are characterized by early appearance of skin carcinoma on exposed areas. We chose to investigate wether a possible DNA repair anomaly exists in cells from patients with this syndrome. This was done by measuring nucleotide excision repair activity, *in vitro* using protein extracts from lymphoid cells, and *in vivo* directly in skin fibroblasts using Unscheduled DNA Synthesis (UDS) detection. For this, we analysed cells from different patients from four distinct families by comparing them with repair efficient cells (healthy patient) and repair deficient cells (XP group A).

The methods used revealed a significant reduction in DNA repair activity (> 40 %) in most patients. Cell survival of Bazex syndrome patients showed significant UVC sensitivity. The complementation of lymphoblastoid extracts with purified XP-A protein, almost completely restored DNA repair activity. Finally, to determine the deficient step in the DNA repair mechanism, we analysed the incision activity of cell protein extracts by using the *in vitro* modified assay. All cells tested were deficient for the incision step.

These repair detection methods demonstrate that the Bazex's syndrome is associated with defective DNA repair particularly at the incision step level. This anomaly would explain the basal cell carcinogenesis seen early on this disease.

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IDENTICAL DELETION OF EXON 87 OF THE COL7A1 GENE IN THREE BRITISH DDEB FAMILIES ARE ACCOMPANIED BY ABNORMAL ANCHORING FIBRIL MORPHOLOGY: A COMMON FOUNDER EFFECT.

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We have previously established linkage to the COL7A1 gene in 6 British families with autosomal dominant dystrophic epidermolysis bullosa (DDEB). Recently we have searched for causative mutations using cultured dermal fibroblast mRNA and RT PCR to amplify cDNA followed by sequencing. By these means we have now identified a deletion of exon 87 caused by a donor splice site mutation (A26314G, accession number: L23982) in 2 of the 6 original families. A further three-generation family with DDEB was ascertained to have this mutation, and subsequently were found to share an ancestral surname with the larger of the 2 original families. Identical mutations and similar clinical phenotypes strongly suggest a common founder mutation in these 3 families who together have 46 affected individuals in 5 generations. Furthermore, ultrastructural analysis for the original 2 families showed both qualitative and quantitative (reduced) changes in anchoring fibrils. We conclude that a common founder mutation causes the same clinical phenotype in these 3 British families. The single autosomal dominant exon skip of COL7A1 significantly impairs anchoring fibril formation.

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COMPOUND HETEROZYGOSITY FOR COL7A1 MUTATIONS IN A FAMILY WITH DYSTROPHIC EPIDERMOLYSIS BULLOSA: A GLYCINE SUBSTITUTION AND AN UNUSUAL SPLICE MUTATION

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Dystrophic epidermolysis bullosa (EBD) is an autosomal blistering disorder of the skin, characterized by loss of dermal-epidermal adherence and by abnormal anchoring fibrils. Here we describe an EBD localisata patient with a heterozygous glycine-to-arginine substitution in the triple-helical domain and a heterozygous 14 bp deletion mutation in an exon-intron 115 boundary of the collagen VII gene, COL7A1. Heteroduplex analysis and sequencing of the PCR product revealed a G-to-A transition in Exon 73 at nucleotide position 6074 of COL7A1 in the proband. This substitution results in the conversion of a glycine to arginine in the -RGD-sequence and was designated as G2009R. Since the parents of the proband were clinically unaffected, this gene defect might be a recessive or a new dominant disease-causing mutation. The second mutation is an exon-intron mutation which led to skipping of exon 115 and elimination of 29 amino acids from the proα1(VII) polypeptide chain. As a result, procollagen VII was not converted to collagen VII, and the C-terminal NC-2 propeptide that is removed from the procollagen under normal conditions, was retained in the skin. The retention of the NC-2 propeptide of collagen VII in one allelic gene product, combined with a glycine substitution in the other allelic product caused destabilisation of the anchoring fibrills and trauma induced dermo-epidermal separation in this patient. The deletion mutation was also found in a second, unrelated EBD family. In addition to the affected individual, several healthy family members carried the mutation. The second family proves that lack of procollagen VII processing does not cause EBD, but a second mutation is required. The nature of the second mutation determines the clinical phenotype.

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HUMAN SOMATIC GENE CONVERSION ACTING AS REVERSE MUTATION IN A PATIENT WITH NON-LETHAL JUNCTIONAL EB.

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A 27-year old female had generalized atrophic benign epidermolysis bullosa, a non-lethal variant of junctional EB, since birth. She had clinically unaffected non-consanguineous parents, seven unaffected siblings, and one affected brother who was deceased. Physical examination disclosed a mosaic skin with symmetrical leaf-like (phylloid) patches over the hands and arms lacking signs of blistering. In these patches blistering could not be evoked, while complementary skin was fragile. IF of fragile skin with antibodies to BP180 was negative. In the mosaic patches, BP180 staining was present in ~50% of the cells in clusters of ~10 cells; other basement membrane components showed normal continuous staining at these sites. Mutation detection using heteroduplex analysis, demonstrated a heterozygous maternal 1-bp deletion (1706delA) in exon 18, resulting in a premature termination codon 54-bp downstream from the deletion. BP180-positive and BP180-negative cells were separated by FACS from keratinocyte cultures established from the mosaic skin. The 1706delA mutation was not present in the BP180-positive keratinocytes, while the mutation was retained in the BP180-negative cells from both mosaic and fragile skin. The reverse mutation in exon 18 was accompanied by conversion of the maternal allele to the paternal allele at the site of a microsatellite marker in intron 18, while these alleles remained heterozygous for a C/T polymorphism in intron 19. The regions flanking the BPAG2 gene on 10q24.3 remained heterozygous, excluding uniparental disomy and gene recombination. The reverse mutation was thus the result of gene conversion and not of gene deletion or back mutation. To our knowledge this is the first reverse mutation in a human in which the mechanism is demonstrated to be gene conversion.

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MUTATIONS IN THE LAMC3 GENE IN NON-LETHAL JUNCTIONAL EPIDERMOLYSIS BULLOSA. D. Castiglia, P. Posteraro, M. Pinola, M. Paradisi, P. Puddu, C. Angelo, L. Gagnoux-Palacios*, G. Meneguzzi*, G. Zambruno. Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy; *INSERM U385, Nice, France.

Junctional epidermolysis bullosa (JEB) is a clinically and genetically heterogeneous autosomal recessive blistering disease of skin and mucous membranes. Nonsense mutations in the genes (LAMA3, LAMB3 and LAMC2) encoding the three laminin 5 chains have been identified in the lethal (Herlitz) variant of JEB. In non-lethal forms, mutations in genes encoding for the β3 and γ2 chains of laminin 5, the 180-kD bullous pemphigoid antigen (BPAG2) and the β4 integrin subunit have been described. The aim of the present study was to identify the molecular defect in a child affected from non-lethal JEB. Immunofluorescence of skin sections showed a reduced expression of all three laminin 5 chains. Northern blot analysis of RNA from cultured keratinocytes evidenced markedly reduced levels of γ2 transcripts. Analysis of the corresponding cDNAs revealed the presence of two distinct mutations in the γ2 gene alleles. The maternal mutation consists of a one base pair insertion (3483insA) resulting in a downstream premature termination codon. The paternal mutation occurs in an acceptor splice site (494-1G→A) and results in in-frame exon skipping involving the amino-terminal domain V which is removed during the extracellular proteolytic processing of the γ2 chain. Immunoprecipitation studies of cultured keratinocyte lysates and medium showed a reduced synthesis of all three laminin 5 chains. Our results indicate that the combination of a nonsense mutation with a mutation leading to in-frame exon skipping upstream to the cleavage site of the γ2 chain may be responsible for the milder clinical phenotype in some cases of non-lethal JEB.

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THE ROLE OF ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL ANALYSIS AS A PRELUDE OR PREREQUISITE FOR THE MOLECULAR DIAGNOSIS OF JUNCTIONAL EPIDERMOLYSIS BULLOSA. John A. McGrath,[1,2] Leena Pulkkinen,[2] James R. McMillan,[1] Angela M. Christiano,[2,3] M. Giles S. Dunnill,[1] Gabrielle Ashton,[1] Jouni Uitto,[2] Robin A. J. Eady.[1] [1]St John's Institute of Dermatology, St Thomas's Hospital, London, UK; [2]Department of Dermatology and Cutaneous Biology, Jefferson Medical College, Philadelphia PA, USA; [3]Department of Dermatology, Columbia University, New York NY, USA.

The autosomal recessively inherited blistering skin disorder, junctional epidermolysis bullosa (JEB), shows considerable clinical and molecular heterogeneity. Thus far, pathogenetic mutations have been delineated in five different candidate genes (LAMA3, LAMB3, LAMC2, BPAG2/COL17A1 and ITGB4). To determine the most appropriate means of elucidating the inherent molecular pathology in patients with JEB, we have undertaken ultrastructural and immunocytochemical studies on skin biopsies from 30 patients with different forms of JEB, in conjunction with mutational screening by PCR amplification of genomic DNA, heteroduplex analysis and nucleotide sequencing. The data showed that electron microscopy was useful in diagnosing JEB as a group of disorders by demonstrating a lamina lucida plane of blister formation, but showed inconsistent perturbations in hemidesmosome morphology in several patients with similar phenotypes. Immunocytochemistry, using specific monoclonal antibodies, was helpful in determining which gene/protein system (laminin 5, 180-kD bullous pemphigoid antigen or β4 integrin) might harbour the mutations. Molecular analysis showed that most of the mutations occurred within the LAMB3 gene, with a preponderance of the mutation R635X in exon 14 of LAMB3 accounting for over 40% of all mutant alleles. These findings suggest that a suitable approach to investigating patients with JEB is to perform initial electron microscopy and immunohistochemical studies to determine the plane of blister formation and to provide evidence for abnormalities in a particular candidate gene/protein system. If laminin 5 is implicated from these findings, then the mutational investigation should first assess the presence or absence of the R635X hotspot mutation in LAMB3, followed by screening of the remainder of LAMB3 and then the other laminin 5 genes.

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GENOMIC ORGANIZATION OF THE SEQUENCES CODING FOR INTEGRIN β4, THE DEFECTIVE GENE PRODUCT IN JUNCTIONAL EPIDERMOLYSIS BULLOSA WITH PYLORIC ATRESIA (PA-JEB). S. Iacovacci, L. Ruzzi, P. Posteraro, G. Zambruno, G. Meneguzzi*, M. D'Alessio. Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy, and *U385 INSERM, Faculté de Médecine, Nice, France.

A recent report has shown that in one PA-JEB patient the disease resulted from 2 distinct mutations in the β4 integrin subunit gene. The β4 protein is an hemidesmosomal component comprised of four structural domains: a 27 aminoacid (aa) signal peptide, an extracellular domain of 710 aa, showing sequence homology to the other integrin β chains, a transmembrane region of 23 aa, and an unusually large cytoplasmic domain of about 1100 aa. In order to facilitate mutation analysis of the gene in PA-JEB, we have determined the structure of the β4 integrin gene, elucidated the exon-intron organization and derived a physical map of the genetic locus. Overlapping genomic clones encoding the human β4 subunit gene were isolated from a phage λ library. The coding portion of the gene spans about 30 Kb and is composed of 35 exons. The exons range in size between 100 and 240 bp and the introns between 64 and 1600 bp. The first 13 exons encode the signal peptide and most of the extracellular domain, the exon 14 encodes the transmembrane domain and the beginning of the cytoplasmic region, while the remainder of the cytoplasmic region is contained in the other 21 exons. This work represents the first comprehensive analysis of the organization of the β4 gene. The β4 gene is structurally related to the other β integrin genes indicating that all these genes duplicated from a common multiexon progenitor.

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DESMIN TRANSFECTION TO REINFORCE THE MUTANT KERATIN IN AN EPIDERMOLYSIS BULLOSA SIMPLEX CELL LINE. SM Morley 1, S Leitgeb 2, HA Nasvaria 3, IM Leigh3, EB Lane1, T Magin 4, 1CRC Cell Structure Research Group, University of Dundee, 2 Institute of Cell and Molecular Biology, University of Edinburgh, 3 Experimental Dermatology Dept, Royal London Hospital, London, 4Institut fur Genetik,Universitat, Bonn.

The inherited blistering disorder, *epidermolysis bullosa simplex* (EBS) is caused by mutations in either of the two keratin intermediate filament proteins K5 or K14 normally expressed in basal keratinocytes. Keratinocytes cultured from patients with EBS show variable keratin filament instability and aggregate formation. Aggregates can be induced in cell lines carrying keratin mutations by a simple temperature shift assay[1]. A possible therapeutic approach to EBS is to introduce an alternative intermediate filament protein such as desmin, into keratinocytes to support the existing network. We have transfected desmin into a keratinocyte cell line KEB -3D (immortalised by SV40 TAg) from an EBS patient with a K14 mutation [2] and compared it with the non-transfected cell line (KEB -3). The presence of desmin did not affect the distribution of the keratin filament network, cell growth or migration in cells grown on plastic dishes. Cells grown in a complex culture system differentiated normally, whilst still expressing desmin. The temperature shift assay demonstrated that keratin filament aggregation was still equally induced in both KEB-3 and KEB-3D, but the desmin filaments remained unaggregated. A recently devised hypo-osmolar shock assay suggests that the increased cell fragility seen in KEB-3 and other EBS cell lines may be reduced in KEB-3D supporting our working hypothesis that the extra intermediate filament does strengthen the cell.

[1] Journal of Cell Science 1995: 108; 3463 - 3471
[2] Nature Genetics 1993: 5; 294 - 300

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PLECTIN MUTATIONS UNDERLIE EPIDERMOLYSIS BULLOSA WITH MUSCULAR DYSTROPHY. Leena Pulkkinen, Frances J.D. Smith, W.H. Irwin McLean, E. Birgitte Lane, Irene M. Leigh, Robin A.J. Eady, John McGrath, Angela M. Christiano, Satoru Murata, Hideo Yaoita, Hiroshi Hatchisuka, Hiroshi Shimizu, Takeji Nishikawa, and Jouni Uitto. Jefferson Medical College, Philadelphia, PA; University of Dundee, U.K.; Royal London Hospital, U.K.; St. John's Institute of Dermatology, London, U.K.; Keio University School of Medicine, Tokyo, Japan.

A distinct variant of epidermolysis bullosa, EB-MD, inherited in an autosomal recessive pattern, demonstrates neonatal skin blistering associated with late-onset muscular dystrophy. Electron microscopy of these patients' skin suggests that tissue separation occurs intracellularly at the level of the hemidesmosomal inner plaque, which contains plectin, a high-molecular-weight cytomatrix protein, also expressed in the sarcolemma of the muscle. We have recently cloned cDNAs which encode the full-length human plectin sequences, and we have elucidated the intron-exon organization of the corresponding gene, PLEC1. In this study, we report four patients with EB-MD, each with homozygous mutations in PLEC1. Immunofluorescence of the skin with HD-1 antibody revealed essentially complete absence of staining. In three cases, the mutations resulted in frameshift and premature termination codon of translation. These findings predict the synthesis of a truncated plectin polypeptide and reduced levels of the corresponding mRNA due to nonsense mediated decay of the mutated transcript. In the fourth family, the proband and had a homozygous 9-bp deletion mutation, designated as 2719del9. This genetic lesion resulted in elimination of three amino acids, QEA, in a stretch of 23 amino acids entirely conserved between the mouse and human sequences. The absence of plectin in the hemidesmosomes, associated with fragility of basal keratinocytes, implicates plectin as critical for binding of intermediate keratin filament network to hemidesmosomal complexes. The function of plectin as a putative attachment protein also in the muscle, mediating binding of the muscle proteins, such as actin, to membrane complexes, would explain the clinical phenotype of EB-MD.

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MORPHOLOGICAL CHANGES IN SKIN AND SKELETAL MUSCLE ASSOCIATED WITH PLECTIN / HD1 DEFICIENCY IN EPIDERMOLYSIS BULLOSA WITH MUSCULAR DYSTROPHY.

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We have recently shown that the level of blistering in the skin of patients with epidermolysis bullosa simplex associated with progressive muscular dystrophy (MD-EBS) is intraepidermal and just above the basal keratinocyte plasma membrane where keratin intermediate filaments normally attach to the inner plaques of hemidesmosomes. In this study we examined skeletal muscle from a patient with MD-EBS together with affected skin and normal muscle as a control. Light microscopy of cross-sectioned MD-EBS muscle showed a variable fibre size with multiple internal nuclei. Immunostaining for plectin (10F6 monoclonal antibody) and HD1 showed a loss of peripheral staining but some retention of 10F6 immunoreactivity within the muscle fibres. Immunostaining for dystrophin and spectrin was similar to that seen in normal controls. Post-embedding immunoelectron microscopy (immuno-EM) localized the 6C6 anti-plectin antibody to the Z-lines of normal muscle and to the cytoplasmic face of sarcolemma-associated densities. These structures might serve as anchorage points for the muscle cytoskeleton, by analogy with hemidesmosomes in basal keratinocytes. This comparison is supported by the immuno-EM localization of both plectin and HD1 to the inner plaque of the hemidesmosome. Transmission EM showed widening of the space between sarcomeres and plasma membrane and a focal reduction, loss or disorientation of Z-lines. A deficiency of plectin would appear to result in defective anchorage of the cytoskeleton with subsequent cell fragility. Genetic defects of dystrophin are also thought to affect normal anchorage of the muscle cytoskeleton.

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CLONING OF THE MURINE PLECTIN cDNA REVEALS HIGH EVOLUTIONARY CONSERVATION OF THIS CYTOSKELETAL ASSOCIATED PROTEIN. Frances J. D. Smith[1], W. H. Irwin McLean[1], Elizabeth L. Rugg[1], Irene M. Leigh[2], Robin A. J. Eady[3], Jouni Uitto[4] and E. Birgitte Lane[1], [1]CRC Cell Structure Research Group, Dundee; [2]Experimental Dermatology, London; [3]St John's Institute of Dermatology, London; [4]Dept Dermatology and Cutaneous Biology, Philadelphia.

Plectin is a high molecular weight cross-linking protein which is involved in many cytoskeletal-membrane interactions, including hemidesmosomes in basal epidermal keratinocytes and the sarcolemma in skeletal muscle. To facilitate transgenic approaches to the study of epidermolysis bullosa with muscular dystrophy (EB-MD), a human autosomal recessive disorder caused by plectin deficiency, we undertook the cloning of the murine plectin cDNA. A cross-species PCR approach was employed, based on the previously reported rat mRNA sequence. About 10 kb of the murine sequence was obtained which showed an overall DNA sequence homology of approximately 94% and protein homology of 98% with the published rat mRNA sequence. The coiled-coil subdomains within the central rod domain which are involved in dimerisation of plectin are highly conserved, whereas the non-helical linker and basic subdomains are not conserved between species. The C-terminal globular repeat domain which is thought to be involved in binding of intermediate filaments is highly conserved between mouse, rat and human plectin sequences, presumably a reflection of the functional importance of this protein domain. Of particular interest is the finding that N-terminal globular domain is very highly conserved between the murine and human genes but is very different from the published rat sequence due to insertions and deletions. A putative transmembrane sequence within this part of the rat cDNA does not occur in the analogous human or murine sequences. Nevertheless, the high degree of homology between the mouse and human sequences suggests evolutionary conservation of the plectin gene.

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EVIDENCE OF GENETIC HETEROGENEITY IN VOHWINKEL'S KERATODERMA. Bernhard P Korge*, Claudia Pünter*, Anthea Stephenson§, and Colin S Munro, Depts. of *Dermatology & Venerology, Univ. of Cologne, Germany, §Human Genetics, Univ. of Newcastle upon Tyne, and Dermatology, Southern General Hospital, Glasgow, UK.

Vohwinkel's keratoderma, characterised by honeycomb keratoderma, pseudo-ainhum and other features including acral "starfish" keratoses, ichthyosis, and deafness, has recently been mapped to the epidermal differentiation complex (EDC) at chromosome 1q21, and a mutation identified in the major cornified cell envelope protein loricrin (J Invest Dermatol 1996;106:811). To investigate further the role of loricrin in Vohwinkel's keratoderma, we have studied 2 new, unrelated families (V1 and V2). All family members were examined and DNA samples were used for linkage studies to 4 microsatellite markers which reside near or within the EDC at 1q21. Members of family V1 (6 cases) had honeycomb or diffuse keratoderma, pseudo-ainhum and a variable ichthyosis, but neither starfish keratoses nor deafness. This phenotype is similar to that of the family in whom a loricrin mutation has been found. LOD scores for D1S498 and D1S1664 were positive (Zmax=1.97 at q=0.0 & 1.69 at q=0.0, respectively), consistent with linkage to 1q21. Currently we are screening the V1 family for a loricrin mutation. Affected members of family V2 (10 cases) had honeycomb keratoderma with pseudo-ainhum, acral starfish and other keratoses, but no ichthyosis. 8 of the 10 had impaired hearing. This family had Vohwinkel's syndrome, as first described. In this family, assuming complete penetrance, linkage was excluded from within 1 centiMorgan of two markers, D1S1664 and D1S305, linked to the loricrin gene within this distance. We conclude that while mutations in loricrin may cause Vohwinkel's keratoderma with ichthyosis but no deafness, the disorder is clinically and probably also genetically heterogeneous.

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FOCAL AND TRANSGREDIENT EPIDERMOLYTIC PALMOPLANTAR KERATODERMA: IDENTIFICATION OF CANDIDATE KERATINS BY IMMUNOGOLD ELECTRON MICROSCOPY. [1,4]O Swensson,[1]R Pitera,[1] WAD Griffiths,[2]L Langbein,[1,3]RC Ratnavel[3], IM Leigh, RAJ Eady. 1-St John's Institute of Dermatology, St Thomas's Hospital, London, U.K. 2-German Cancer Research Centre, Heidelberg, Germany; 3-Experimental Dermatology Lab., London Hospital, U.K. 4-Dept of Dermatology, University of Kiel, Germany.

Keratin 9 gene mutations have been identified as the underlying genetic defect in epidermolytic palmoplantar keratoderma (PPK) of Vörner. To see whether keratins represent candidate proteins in other forms of PPK we performed an ultrastructural study including postembedding immunogold electron microscopy in autosomal-dominant transgredient (n=3) and focal (n=3) forms of PPK.

The keratin filament network of lesional ridged epidermis was unconspicious in two families with transgredient PPK. In the third family affected members showed abnormal keratin aggregates in lesional suprabasal cells. In focal PPK all affected individuals studied showed abnormal keratin clumping mostly confined to the upper spinous and granular cells. These changes could not be identified reliably at the light microscopical level but were evident by electron microscopy. Keratin aggregates present in lesional ridged skin of transgredient and focal forms of PPK labelled with several anti-keratin antibodies includuing AM1 (K9), KA12 (K6), and LL025 (K16).

Our findings show that ultrastructural changes of epidermolytic hyperkeratosis are not confined to PPK of Vörner but can be seen in forms of transgredient and focal PPK. Abnormal keratin aggregates were regularly seen in focal PPK, whereas epidermolytic and non-epidermolytic forms were noted in transgredient PPK. Our immunolabelling data indicate the involvement of keratins 9, 6, and 16 in the formation of keratin aggregates in both focal and epidermolytic transgredient PPK. These keratins should be considered candidate proteins and genes in these disorders.

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LINKAGE STUDIES IN ERYTHROKERATODERMIAS: FINE MAPPING, GENETIC HETEROGENEITY, AND ANALYSIS OF CANDIDATE GENES. G. Richard, Y.M. Whyte, L. Smith, P. Itin*, D. Hohl**, U. Wollina#, E. Epstein, Jr.##, J.P. Lin, S.J. Bale, Laboratory of Skin Biology, NIAMS, NIH, Bethesda, MD; Departments of Dermatology, *University Basel, CH; **Hospital Beaumont Lausanne, CH; #University Jena, Germany; ##General Hospital, University San Francisco, Ca.

Erythrokeratodermias are a clinically heterogeneous group of rare inherited disorders of cornification characterized by two distinct morphological features: hyperkeratosis and erythema. We ascertained two large families segregating autosomal dominant erythrokeratodermia variabilis (EKV) and one family with Greither's palmoplantar keratoderma/EKV phenotype for a linkage study. The phenotype in the two EKV families maps to chromosome 1p32-34 with a maximum multipoint lod score of 6.53 between D1S496-D1S1591, approximately 15cM centromeric to the RH locus. In contrast, in the third family with 8 affected members having Greither's PPK, of which 2 also display EKV, this region was completely excluded with lod scores less than -2 for 20cM. These results indicate different genetic defects in the EKV and Greither's PPK/EKV families.

Based on recombinations in the two EKV families, we excluded 4 genes assigned to 1p: Gardener-Rashed feline sarcoma oncogene, cartilage matrix protein, avian myelo-cytosis viral oncogene, and neuronal ceroid lipofuscinosis. Two members of the connexin gene family, Cx37 and Cx40, encoding GAP-junction proteins map within the EKV region, are expressed in keratinocytes, and are therefore attractive candidate genes. Direct sequencing of the coding region of Cx37 in EKV patients revealed several variations, including a novel polymorphism within the 5' cytoplasmic domain of Cx37, which did not cosegregate with the disease. No pathogenic mutations were found, thus excluding Cx37 as a candidate gene for EKV. However, there is evidence that other epidermally expressed connexins cluster in this region, and one may yet be determined to play a role in the pathogenesis of EKV.

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CELL RECEPTOR MEDIATED DNA TRANSFECTION REVERTS THE ENZYME DEFECT IN LAMELLAR ICHTHYOSIS CELLS. M. Huber, N. Burri, E. Wagner*, and D. Hohl, Dept. of Dermatology, University Hospital, Lausanne, Switzerland, and *Bender Institute, Vienna, Austria.

The finding of mutations in the keratinocyte transglutaminase (TGK) gene in some autosomal recessive lamellar ichthyosis (LI) patients together with the suprabasal location of the deficiency facilitate a topical gene therapy approach for this disease. As a first step towards this goal, cultured (high calcium) TGK-deficient keratinocytes (activity in membrane ≤ 200pmol/mg hour) were transfected with a wildtype TGK cDNA construct controlled by the CMV I/E promoter or the human involucrin promoter. For transfection either the adenovirus-enhanced transferrin receptor system or lipofectin was used. Two and five days after transfection transglutaminase (TG) activity was measured in the membrane fraction (n=3). TG activity reached a maximum of 3880 pmol/mg hour in the membrane with the cell receptor-mediated system at two days. Five days after transfection membrane TG activity was reduced to 1600 pmol/mg hour. In cultured cells of normal individuals and heterozygotic relatives of patients membrane TG activity was in the range of 1500-6800 pmol/mg hour. Transfections of the construct with the involucrin promoter using the receptor-mediated system yielded about 10 fold lower membrane TG activities. Cells transfected using lipofectin showed no significant increase in their TG activity levels. These results indicate that DNA delivery using a cell surface receptor based system restores normal enzyme levels in cultured TGK-deficient keratinocytes. Further experiments will show if in vivo topical DNA application using the receptor based system normalizes TG activity and reverts the phenotype.

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UNDER-REPRESENTATION OF THE K10 MUTANT ALLELE IN A PATIENT WITH MOSAIC EPIDERMOLYTIC HYPERKERATOSIS.

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Epidermolytic hyperkeratosis (EH or CBIE) is an autosomal dominant genetic disorder that has full penetrance and is due to mutations in keratins, K1 or K10. Linear epidermal naevus (LEN) represents a mosaic form of EH where mutation bearing keratinocytes migrate over the skin surface, often following Blaschko's lines. We have examined patients with EH or LEN, and found that even in the affected skin of some LEN patients, the keratin mutation does not fully penetrate and the mutant allele is under-represented.

Peripheral blood and biopsies of affected skin were taken from patients with LEN (CM, AE) or EH (DT) and normal skin of LEN patients. Epidermal keratinocytes were cultured to confluence (12 days) and genomic DNA or total RNA extracted. The helix encoding regions of K1 and K10 were amplified by PCR and sequenced. Mutations in the 1A domain of K10 were found in two patients (R156S in DT and R156H in CM) but no sequence alteration was found in AE even though affected skin showed typical EH histopathology. However, RFLP analysis showed an altered Aci I site in exon 1 of the K10 gene from affected cells but the mutant allele was under-represented (mutant:normal 1:3 not 1:1 as expected) and not detectable by direct sequencing. Thus, patients with mosaic EH do not necessarily have 100% of mutation bearing keratinocytes in skin that shows typical corrugate hyperkeratosis. We are now cloning the K10 mutant allele to identify the precise base change in this LEN patient.

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FURTHER REFINEMENT ON CHROMOSOME 9q OF THE NEVOID BASAL CELL CARCINOMA SYNDROME GENE. Fabio Magrini, Ketty Peris, Robert A Furlong[§], Maria Concetta Fargnoli, Sergio Chimenti. Department of Dermatology, University of L'Aquila, Italy and [§]Department of Pathology, University of Cambridge, U.K.

Nevoid Basal Cell Carcinoma Syndrome (NBCCS) is an autosomal dominant disorder clinically characterized by basal cell carcinomas of the skin, ovarian fibromas, medulloblastomas and widespread developmental defects. The NBCCS gene has been recently mapped to 9q22.1-q31 between D9S196 and D9S180. We examined a NBCCS patient for loss of heterozygosis (LOH) at nine microsatellite loci (cen-D9S197, D9S196, D9S280, D9S287, D9S180, D9S176, D9S109, D9S127, GSN -tel) located in 9q22.2-q31. LOH was found at D9S287, D9S180 and D9S109, but not at D9S176 that lies between D9S180 and D9S109. Our results suggest that the NBCCS gene is placed distal to D9S287 and proximal to D9S180, while the deletion at D9S109 is likely to be due to a somatic event not affecting the NBCCS gene. In addition, we studied germline DNA from three descendants of this patient affected with NBCCS but without skin tumors. In the interval between D9S287 and D9S109, the alleles conserved after LOH were always inherited by these individuals. The loss of the nontransmitted alleles as observed in our patient, strongly supports the hypothesis that NBCCS is due to mutation in a tumor suppressor gene important in the development of the embryo as well as in normal cell growth and differentiation.

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EVIDENCE FOR GENETIC HETEROGENEITY IN BASAL CELL CARCINOMA SYNDROME AND FURTHER NARROWING OF THE LOCUS ON CHROMOSOME 9 q

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The nevoid basal cell carcinoma syndrome is an autosomal dominant disorder characterized by predisposition to multiple early basal cell carcinomas of the skin and several other tumors as well as frequent occurrence of developmental anomalies. The gene has been mapped to chromosome 9q22 and is believed to function as a tumor suppressor. We have applied linkage and haplotype analysis to five Swedish nevoid basal cell carcinoma syndrome families to refine the localization of the nevoid basal cell carcinoma syndrome gene. Information from critical recombinants localizes the gene between D9S180 and D9S196 and in combination with analysis of loss of heterozygosity in a hereditary cardiac fibroma we found a minimal candidate region of 1Mb or less for the nevoid basal cell carcinoma gene flanked by the markers D9S280 and D9S287 in the 9q22.3 area. Results obtained from one family with typical clinical findings provide strong evidence for non-linkage to 9q22.3 and thus for the first time implicate the occurrence of genetic heterogeneity in nevoid basal cell carcinoma syndrome.

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ALTERATIONS OF MICROSATELLITES ARE A FEATURE OF MUIR-TORRE SYNDROME. Ketty Peris, Maria Teresa Onorati, Sergio Chimenti. Department of Dermatology, University of L'Aquila, Italy

The Muir-Torre syndrome (MTS) is an autosomal dominant genodermatosis characterized by the presence of sebaceous gland tumors (adenoma, epithelioma or carcinoma) associated with at least one internal malignancy. Recently, microsatellite instability (MSI) has been demonstrated in cutaneous and visceral tumors of some patients with MTS. In addition, mutations of the mismatch repair gene MSH2, located in the region of D2S123, have been identified in MTS and Hereditary nonpolyposis colon cancer (HNPCC). We examined sebaceous gland tumors and visceral malignancies from two unrelated patients with MTS for microsatellite instability and loss of heterozygosity (LOH) at chromosomes 2p, 3p, 5q, 9p, 17p and 18q. MSI was observed in 4 of 4 skin tumors (2 sebaceomas, 1 adenoma and 1 carcinoma) at 2p22 (D2S123), 3p21.2-21.3, 5q21, 9p21, 17p12-11.1 and 18q23 whereas LOH was identified at 2p22 (D2S119) in 2 of 4 tumors (1 adenoma and 1 carcinoma). Furthermore, the colon carcinoma displayed MSI at 17p12-11.1 and LOH at 2p22 (D2S123).

Our results further support the evidence that MSI is a feature of sebaceous gland tumors in MTS patients. In addition, the finding of LOH at D2S123 locus on chromosome 2p in the colon carcinoma suggests that: 1) defective mismatch DNA repair gene(s) (e.g. MSH2) could play a pathogenetic role in MTS, 2) in a proportion of patients, MTS and HNPCC may share a commongenetic basis.

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MULTIPLE MICROSATELLITE ALTERATIONS ON CHROMOSOME 9 IN NEUROFIBROMATOSIS TYPE 1. MC Fargnoli, K Peris, A Antonelli*, F Magrini, S Chimenti. Department of Dermatology, University of of L'Aquila, Italy. *Department of Genetics, University of L'Aquila, Italy.

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with a highly variable phenotypical expression. The major clinical features include peripheral neurofibromas, café-au-lait spots, axillary and inguinal freckling, Lisch nodules of the iris, and an increased risk of malignancy. NF1 is caused by germ-line mutations of the NF1 gene, which has been mapped to chromosome 17q11.2 and encodes for neurofibromin, a member of the GTPase-activating protein superfamily of p21*ras* regulatory proteins. Microsatellite instability (MSI) was first described as a key feature of hereditary nonpolyposis colon cancer (HNPCC). Since it has also been detected in several sporadic or familial neoplasms, we evaluated MSI in neurofibromas from 2 NF1 patients, father and daughter. Two intronic markers of the NF1 gene (IVS27AC28.4 and IVS38GT53.0) and 16 microsatellite loci scattered throughout the genome (D2S119, D2S123, D3S1317, D3S1611, D5S107, D5S346, TFIID, D9S109, D9S126, D9S127, D9S144, D9S176, D9S196, D9S287, D17S261, D17S520) were analyzed on DNA extracted from peripheral leukocytes and formalin-fixed, paraffin-embedded tissue sections of 3 neurofibromas of the father and one of the daughter. MSI was detected in 3 out of 3 neurofibromas of the father at D9S126 and in two out of 3 at D9S176 and D9S196. One of these tumors showed additional alterations at D9S127, D9S287 and D2S123. No MSI was identified in the neurofibroma of the daughter. Our data confirm that MSI is a common genetic event in neurofibromas of NF1 patients. In addition, the observation of MSI at multiple loci on chromosome 9 as well as at D2S123, which is close to the mismatch repair gene MSH2 that is known to be involved in the pathogenesis of HNPCC, suggests that "modifying genes" located within these chromosomes might have a role in the pathogenesis of NF1-associated neurofibromas.

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HAILEY-HAILEY DISEASE: STRUCTURAL ALTERATIONS IN LESIONAL AND NON-LESIONAL SKIN.

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Hailey-Hailey disease is a blistering genodermatosis that has been successfully treated by dermabrasion. The aim of our study was to investigate the structural alterations of the epidermis in Hailey-Hailey patients before and after surgical treatment. Cryosections were stained for desmosomes, adherens junctions, actin filaments and actin associated proteins and investigated with a confocal laser scanning microscope (CLSM). Hailey-Hailey lesions started with a suprabasal clefting evolving into acantholytic dyskeratosis throughout the epidermis. Incomplete acantholysis was also detectable in clinically unaffected skin. Additionally, clefting at the margins of punch biopsies reflected the reduced cellular cohesion of keratinocytes in Hailey-Hailey patients. Although keratinocytes showed internalisation of desmosomes and perinuclear collapse of the keratin cytoskeleton they were partially linked together by well preserved adherens junctions. Staining for actin filaments with phalloidin proved a remarkable formation of actin stress fibers in response to the tension generated across the adherens junctions. The lower portions of the hair follicles and sweat ducts never were affected by the intrinsinc defect of cell adhesion. The effective therapy with dermabrasion depended on a sufficient eradication of the surface epidermis. Accordingly, reepithelialization from adnexal structures resulted in an intact epidermis not susceptible to blistering. In conclusion, incomplete acantholysis as demonstrable in both lesional and non lesional skin of Hailey-Hailey patients appears to be based on the compensatory function of the actin-adherens junction system.

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REFINED LOCALISATION OF THE DARIER'S DISEASE AND SPINOCEREBELLAR ATAXIA TYPE 2 GENES AT CHROMOSOME 12q24.1; CONSTRUCTION OF A YAC CONTIG ENCOMPASSING THESE GENES. Steven D Bryce*, Simon A Carter*, Joanne T Pang[†], Rebecca Twells[†], Beatrice Renault[‡], Bruno Schleich-Norte[†], Rebecca Allotey[†], Colin JD Ross*, Eleni Katsantonia*, J Weissenbach[#], Raju S Kucherlapati[‡], Jonathan L Rees[¶], Colin S Munro[§], Susan Chamberlain[†], Tom Strachan*. Departments of *Human Genetics & [¶]Dermatology, University of Newcastle Upon Tyne, [§]Dermatology, Southern General Hospital, Glasgow, [†]Biochem. & Molecular Genetics, St Mary's Hospital, London, UK, & [‡]Molecular Genetics, Albert Einstein College of Medicine, New York, USA, and [#]Généthon, Evry, France.

Darier's Disease (DAR) and a locus (SCA2) for the neurological disorder spinocerebellar ataxia are both linked to the same region of human chromosome 12 (*Genomics* 1994;24:378 & 1995;25:433). To further the positional cloning of genes for these disorders, we have used additional microsatellite markers to study crossover events in families with DAR and SCA2. We have also developed and ordered a series of DNA fragments for the region under study.

Definitive crossover events establish flanking markers for the DAR gene, D12S1645 and D12S809. The critical region for SCA2 lies in the same segment, between the markers D12S1328 and D12S1333. A contig of clones has been constructed which spans the region between D12S105 to D12S1329, entirely covering the critical region for SCA2 and almost all of that for DAR. The contig comprises over 40 YAC and several PAC clones and includes more than 50 sequence-tagged site markers.

We conclude that DAR & SCA2 genes are very closely linked. The YAC contig will be valuable in determining the separate gene defects responsible for these conditions.

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A NOVEL MUTATION IN THE LYSYL HYDROXYLASE GENE IN A PATIENT WITH EHLERS DANLOS-SYNDROME TYPE VI. J. Brinckmann* S. Kügler#, Y. Acil#, E. Katzer*, S. Feshchenko', V. Sokolnik', P. K. Müller# and H. H. Wolff *, *Department of Dermatology, #Institute for Molecular Biology, Medical University, Lübeck, D; 'Byelorussian Institute for Hereditary Diseases, Minsk, BY.

The Ehlers Danlos-Syndrome type VI (ED VI) is an autosomal recessive inherited disorder of collagen metabolism characterized by kyphoscoliosis, joint hypermobility and skin fragility. The underlying defect is a mutation in the gene coding for peptidyl lysyl hydroxylase (PLOD) resulting in a low hydroxylysine content in mature collagen.

cDNA and genomic DNA of fibroblasts from a patient with ED VI as well as genomic DNA from the healthy family (parents and one sister) were amplified by (RT)-PCR. Single strand conformation polymorphism (SSCP) analysis was performed with fragments of the PLOD gene containing the exons 13-18 and 16-19.

SSCP analysis of a cDNA fragment from the patient showed a band shift. A homozygote point mutation 2036 G→C was detected by sequencing of this region resulting in a loss of a restriction site for Mwo I. Restriction analysis of genomic DNA from the patient and from the family showed a heterozygote mutation for the patient and his mother, whereas no mutation was found for the healthy father and sister.

Our data showed a novel heterozygote mutation in the PLOD gene as a combination of a point mutation (2036G→C) and a null allel. The mutation results in a substitution of Trp_{612}→Cys in the gene product. The residues 570-709 in the carboxy-terminal region of PLOD are highly conserved (94% homolgy) suggesting that this region contains functional significant sequences.

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A NEW MUTATION OF THE WILSON DISEASE GENE IN A PATIENT WITH LOW LYSYL OXIDASE ACTIVITY IN CULTURED SKIN FIBROBLASTS AND ELASTOSIS PERFORANS SERPIGINOSA

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Wilson disease (WD) is a rare autosomal recessive disease. Two Finnish patients with WD were investigated. The 53-year-old WD patient had clinically elastosis perforans serpiginosa (EPS) and histology revealed abnormal elastin characterized as irregular fibers and lateral budding, "lumpy-pumpy" appearance. Elastin abnormality was found also in the normal looking skin. The patient had been on the D-penicillamine treatment for over 30 years. The activity of lysyl oxidase (LO), a copper-dependent enzyme catalyzing the crosslinking of elastin and collagen, was markedly reduced in cultured skin fibroblasts. In contrast, LO activity was normal in the cultured skin fibroblasts of the 22-year-old WD patient and a third patient with rheumatoid arthritis who had both treated with D-penicillamine. The genetic mutations of the WD patients were investigated using single-strand conformation polymorphism (SSCP) analysis and DNA sequencing. The 53-year-old WD patient had a mutation at exon 4, which converted the codon of CAG for glutamine at position 544 to-TAG, a codon for termination. The mutation occurred in both alleles, indicating that the patient was homozygous. This mutation has not been described in the Wilson gene so far. The other patient had a missence mutation in exon 14 and the previously described deletion, 3809del6, in exon 17, indicating that the patient was a compound heterozygote. No Wilson gene mRNA could be found in cutured skin fibroblasts. The causal relationship between low lysyl oxidase activity and the mutation of the WD gene in the 53-year-old patient is currently unknown.

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EVIDENCE FOR A NEW TYPE II HAIR SPECIFIC KERATIN GENE AND ITS POSSIBLE ROLE IN GENETIC HAIR DISORDERS. Paul E. Bowden and David O. Jones. Dept. of Dermatology, University of Wales College of Medicine (UWCM), Cardiff, UK.

Hair-specific keratins are members of the IF multigene family whose expression is restricted to hair, nail, tongue and thymus. Ten have been described at the protein level (Hb1-4, Hbx, Ha1-4, Hax) together with two additional hair related keratins. Mutations within conserved regions of keratin genes have been identified in several genetic disorders of the epidermis, oral epithelia and nail. Recent evidence has also shown linkage of the genetic hair disorder, monilethrix, to the keratin gene cluster on chromosome 12q. In our genome wide search for hair keratin genes, we have found a type II gene that has unique C-terminal sequence and therefore not one of the known human type II hair keratin genes.

We screened a human cosmid library with PCR generated probes to type I and type II hair-specific keratin sequences. We identified known genes by PCR of exon 9 and 3'nc sequences and isolated unique keratin clones. One clone isolated on a 35 Kb Kpn I fragment encoded exons 1-9 of a type II hair-specific keratin gene. The gene spanned 7.6 Kb on four Hind III fragments (1.6 Kb, 1.65 Kb, 1.37 kb, 10.5 Kb). The C-terminal and 3'non-coding sequences of this gene were closely related to other cortical hair-specific keratin genes (Hb1, Hb3 and Hb4) but were unique. This data provides evidence for another type II hair-specific keratin gene and we are currently investigating the expression of this gene in the hair follicle and its possible role in monilethrix.

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HOMOZYGOSITY FOR HLA-DR RELATED TO FRECKLES

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Homozygosity for HLA antigens has been reported to be a risk factor for the development of several kinds of cancer, including skin cancer. Freckling in childhood and/or adolescence was found to be an independent risk factor for the development of skin cancer.

We undertook a population-based survey on skin cancer among the white inhabitants of Saba, an island of the Dutch Antilles, in 1994 and unexpectedly found a strong association between homozygosity for HLA-DR and freckles. To confirm this association in the Netherlands we undertook a study in a population of healthy blood donors of the bloodbank of Leiden. All these subjects were allready typed for HLA antigens. Unlike the study at Saba, now freckles and lentigines were separately assessed.

The association between homozygosity for HLA-DR and freckles found at Saba (odds ratio 4.3; 95% confidence interval 1.2 to 15.5) was confirmed in a much stronger way in Leiden. All 10 subjects homozygous for HLA-DR had freckles (odds ratio indeterminately high; 95% confidence interval 4.5 to infinite) whereas only 28 of the 66 subjects heterozygous for HLA-DR had freckles.

The relevance of the association between homozygosity for HLA-DR and freckling needs to be determined. Possibly, freckles harbor mutated melanocytes which can be less effectively cleared by subjects homozygous for HLA class II antigens.

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GENETIC PREDISPOSITION LINKED WITH HLA CLASS 2 ALLELES IN ACUTE GENERALISED EXANTHEMATIC PUSTULOSIS (AGEP) AND MACULOPAPULAR RASH (MPR).

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Cutaneous drug reactions are commonly supposed to result from a mediated immune reaction, especially type IV hypersensitivity. The likelihood of T cells implication lead us to a search for a genetic predisposition in AGEP (a rare clinical variant of drug reaction) and MPR (the most frequent drug rash) from a prospective study of 39 patients.

HLA class 1 alleles were identified by microlymphocytotoxicity and HLA class 2 alleles by DNA typing (PCR-SSP). Results were compared to those of a regional reference population. Without taking into account the culprit drug, we observed different significant HLA associations in AGEP (DRB1*11, DQB1*0302) and MPR (DRB1*1301, DQA1*0103, DQB1*0603). Furthermore, when we analyzed each type of drug reaction according to the culprit drug, a highly significant HLA correlation was found for MPR induced by antibiotics other than pristinamycin with the haplotype DRB1*1301, DQA1*0103, DQB1*0603 with a relative risk (RR) of 6 and DRB1*04 (RR=4.2); and for AGEP induced by non antimicrobial drugs with HLA DQA1*0103 (RR=7.1).

These data, obtained using molecular genetics methods, support the hypothesis of involvement of a T cell-mediated immune response with qualitative differences between MPR and AGEP. Interestingly, this study also suggests that the observed immunogenetic predisposition of these two types of cutaneous drug reaction probably differs according to the culprit drug class.

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LOSS IN CELL-CELL CONTACTS ASSOCIATED WITH A CHANGE IN β-CATENIN PHOSPHORYLATION INDUCED BY OKADAIC ACID AND CALYCULIN A IN HUMAN KERATINOCYTES

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Phosphorylation and dephosphorylation events involved in post-translational modifications of junctional elements may critically control junction assembly and stability, and regulate the formation of the cadherin-cytoskeleton complex, thus influencing the adhesive function of cells.

In the present study, we have used specific activators and inhibitors of protein kinases and phosphatases, in a combined biochemical and morphological approach, to analyse the role of protein phosphorylation in the maintenance of epithelial architecture. Okadaic acid and calyculin A, two potent inhibitors of serine-threonine protein phosphatases, dramatically modified the keratin-cytoskeleton of epidermal cells and led to a loss of cell-cell interactions. This effect was not tissue and species restricted. The observed changes were highly specific corresponding to the inhibition of protein phosphatase 1. They were time- and dose-dependent, and reversible excluding a slight cytotoxic effect of the drugs. A decrease in electrophoretic mobility of β-catenin was observed in treated cells, suggesting a change in the protein phosphorylation level and/or protein conformation. The ^{32}P labeled phosphoaminoacids analysis demonstrated that β-catenin was exclusively phosphorylated on serine-threonine residues but not on tyrosine residues, as was previously reported. Immunoprecipitations and western blotting using anti-phosphoserine and anti-phosphotyrosine antibodies confirmed these data.

Here, we reported for the first time that okadaic acid and calyculin A induced not only a drastic change in cell phenotype, but also a loss in cell-cell contacts in primary human cultures of normal keratinocytes and fibroblasts as well as in skin explants. These results may explain the high toxic effect of these drugs and confirm the main role of β-catenin phosphorylation in the regulation of cell-cell contacts.

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ACTIVATION-DEPENDENT HYALURONATE (HA) BINDING BY HUMAN MONOCYTES (Mo): RELEVANCE FOR MIGRATION INTO SITES OF CUTANEOUS INFLAMMATION.

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During cutaneous inflammation activated Mo migrate into inflamed skin where they interact with ECM components like HA, produced in high amounts at inflammatory sites. To date three cellular HA receptors, the variably spliced glycoprotein CD44, ICAM-1 and RHAMM, have been identified. We whished to determine whether 1) activation affects the capacity of Mo to interact with HA and 2) which receptor mediates such binding. Mo freshly purified from peripheral blood (fMo) or Mo subjected to tissue-culture +/- IFN-γ or LPS stimulation (cMo) were analyzed by FACS using mAbs against CD14, different epitopes within the standard portion of CD44 (CD44s), sequences encoded by CD44 variant exons v3-v10, ICAM-1, RHAMM and FITC labelled-HA. fMo bound little HA-FITC, expressed CD44s, but little or no CD44v3-v10, ICAM-1 and RHAMM. By contrast, cMo upregulated their HA binding capacity, ICAM-1, CD44s, CD44v3-v9, all of which were further augmented by IFN-γ or LPS, whereas RHAMM was uninducible. Lymphocytes from the same donor did not bind HA irrespective of stimulation. LPS or IFN-γ-induced HA binding was inhibited by >90% with mAbs directed against the HA binding domain of CD44s but not by a panel of mAbs against other epitopes of CD44s, CD44v3-v10 or ICAM-1. Disruption of CD44 N-linked-glycosylation by tunicamycin inhibited IFN-γ-induced HA-binding most likely by downmodulating the expression of HA-binding domains of CD44s, as detected with mAb-staining. At sites of inflammation, but not in normal skin, activated infiltrating MO displayed high HA-avidity and expressed CD44s, CD44v3-v9, ICAM-1, but no RHAMM, while infiltrating lymphocytes or keratinocytes bound little if any HA, as detected by multicolor immunofluorescence. In conclusion, we show that 1) upon in vitro or in vivo activation monocytes enhance markedly their capacity to bind HA, 2) this is critically dependent upon the expression of CD44-isoforms containing distinct HA-binding domains but not of other HA-receptors or the deglycosylation of CD44. Such regulated CD44-HA interactions may be important for the ability of Mo to migrate into and within sites of cutaneous inflammation.

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NOVEL ANTI-INFLAMMATORY PROPERTIES OF ERYTHROMYCIN: *IN VITRO* REDUCTION OF MONOCYTE INTERCELLULAR ADHESION MOLECULE-1 EXPRESSION

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Peripheral blood mononuclear cells (MNC) require upregulation of LFA-1 and ICAM-1 adhesion molecules to progress to inflammatory tissue loci. We have examined the effects of erythromycin (EM) on LFA-1 and ICAM-1 expression by human MNC cells *in vitro*.

Data from direct immunofluoresence microscopy showed that EM (5 and 50μg.ml^{-1}) had no significant effect on LFA-1 expression on MNC from 6 healthy volunteers, after stimulation for up to 48 hours with PHA or antigen (*P.acnes*). However, measurement of ICAM-1 was found to be variable and was further investigated by FACs analysis of MNC from three volunteers. ICAM-1 expression after 24 hours by lymphocytes was very low. EM had no effect at 40-80μg.ml^{-1}, but at 100μg.ml^{-1} significantly reduced ICAM-1 expression levels (Control 24±2; 100μg.ml^{-1} EM 21±2, $P<0.01$; ANOVA). Expression of ICAM-1 on monocytes from each volunteer was constitutively high, but was significantly reduced at concentrations of EM above 50μg.ml^{-1} (Control 569±97; 60μg.ml^{-1} EM 200±235; 80μg.ml^{-1} EM 241±184; 100μg.ml^{-1} EM 213±67; $P<0.01$).

This study demonstrates a mechanism by which erythromycin could reduce early inflammatory cell interactions and thus modulate tissue inflammation.

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UVA and leukocyte-endothelial interactions: a question of dose and conditions of UV-exposure.

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Ultraviolet radiation (UVR) displays profound effects on the expression of cell adhesion molecules (CAM) resulting in altered cell-cell-interactions. This mechanism is of fundamental importance to understand the diversity of adverse (pro-inflammatory) and therapeutic (anti-inflammatory) skin reactions induced by UVR. High dose polychromatic UVA (90 J/cm^2, maximal emission at 375 nm) induced vascular CAM-expression, particularly of ICAM-1 and E-selectin in vivo (skin type II) as demonstrated by immunohistochemistry. This effect appears to be independent of epidermis-derived mediators as isolated dermal microvascular endothelial cells (DMEC) displayed the same response in vitro demonstrated by FACS-analysis when irradiated with 30 J/cm^2 UVA which is an equivalent exposure dose considering UVA-penetration of 30-50% to the dermal vascular bed. In contrast to a single exposure, endothelial CAM-expression returned to baseline after repetitive (>5) daily UVA-exposures to the same dose. Moreover, pro-inflammatory cytokine challenge (up to 100 U/ml TNFa, IL-1a, IFN-g or a combination thereof) resulted in considerably weaker induction of ICAM-1 (55%) and even more so VCAM-1 (35%) compared to non-irradiated but cytokine stimulated controls. This reduced cytokine responsiveness could also be achieved by lowering daily UV-doses down to 5 J/cm^2. The resulting endothelial-leukocyte interaction was monitored in a newly developed cell adhesion assay. Differential quantification of peripheral blood derived granulocytes, lymphocytes and monocytes revealed reduced adhesion most severely in lymphocytes (32%) followed by monocytes (55%) and granulocytes (68%) compared to leukocyte adhesion to non-irradiated but cytokine stimulated DMEC. We conclude: UVA can substantially influence endothelial CAM expression and thus directly interfere with leukocyte-endothelial cell adhesion. Divergent UVA-induced effects in this respect can be attributed to the mode of UV-exposure as well as the condition of endothelial cells prior to exposure. UVA-doses as low as 5 J/cm^2 are sufficient to exert an anti-inflammatory effect on stimulated DMEC.

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SELECTIVE INHIBITION OF E-SELECTIN EXPRESSION ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC) BY FK506, BUT NOT BY CYCLOSPORIN

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Expression of adhesion molecules on endothelial cells (EC) is of primary importance for the quality and quantity of leukocyte migration towards inflammatory sites. Peripheral blood leukocytes mainly bind to EC via E-selectin, ICAM-1 and VCAM-1. Since these are principal adhesion molecules on skin EC their modulation by pharmacologic agents could play a role in anti-inflammatory treatment strategies.

In the present study we asked the question whether the immunosuppressive agents FK506 and cyclosporin (Cy) may be able to modulate the expression of E-selectin, ICAM-1, and VCAM-1 on HUVEC.

HUVEC were isolated and cultured by standard procedures. FK506 and Cy as well as its solvent ethanol were added to HUVEC-cultures stimulated by rIL-1ß for 1 and 4 hours and compared to the medium control. Expression of adhesion molecules was determined by incubation of HUVEC with specific monoclonal antibodies conjugated with peroxidase and the reaction product was measured photometrically.

The results of this study show a dose-dependent inhibition by FK506 (0.01 to 1 µg/ml) of E-selectin expression on IL-1ß-stimulated HUVEC. Cy (1 µg/ml) also induced a significant decrease of E-selectin expression, however, its solvent ethanol showed the same effect. FK506 and Cy did not change the expression of ICAM-1 and VCAM-1 on HUVEC after IL-1ß stimulation.

We conclude from our results that the expression of E-selectin on HUVEC is selectively inhibited by FK506. Since Cy did not show this inhibitory activity both drugs, although sharing common immunosuppressive pathways, may exert differential effects on adhesion molecule expression in HUVEC.

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RT-PCR FOR E-SELECTIN: A NEW TOOL TO INVESTIGATE THE ANTIINFLAMMATORY POTENCY OF STEROIDS IN VIVO?

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Irritation of the epidermis is an established model to study the dynamics of inflammation. Our previous studies have shown that mRNA of E-selectin, necessary for adhesion of leukocytes, is rapidly upregulated after irritation and can be monitored by RT-PCR. There is a current need to monitor the antiinflammatory potency of topically applied drugs in vivo. Therefore the present study was aimed to analyze the effect of topically applied steroids and subsequent irritation with sodiumdodecylsulphate (SDS) on the regulation of the mRNA level of E-selectin. We investigated 40 biopsy specimen of ten healthy volunteers with the following four test conditions: Group I (no pretreatment, no SDS 1%), Group II (no pretreatment, SDS 1%), Group III (pretreatment with basic ointment, SDS 1%), Group IV (pretreatment with prednicarbate 0,25%, SDS 1%). For Group I, we found a weak positive E-selectin signal in 1/10 biopsies, for Group II a strong positive signal in 10/10 biopsies, for Group III a positive signal for E-selectin in 10/10 biopsies, in Group IV a decreased E-selectin signal in 9/10, but no complete elimination of the signal. Our study shows for the first time that topically applied corticosteroids have a clear-cut effect on the early mRNA upregulation of E-selectin. This method should be proved to be a useful tool to study the antiinflammatory effect of topically applied pharmacological substances in vivo.

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EFFECT OF LEUKOTRIENE B4 ON THE EXPRESSION OF ADHESION MOLECULES IN CULTERED HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS (HDMEC).

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The arachidonic acid metabolite leukotriene B_4 (LTB_4) induces chemotaxis, chemokinesis, aggregation and degranulation of neutrophils as well as neutrophil adherence to endothelial cells. Topical application of LTB_4 on the skin causes an inflammatory reaction characterized by intra-epidermal neutrophil microabscesses and is used as a model for evaluating the effectiveness of treatment modalities in psoriasis. LTB_4 has been reported to induce a transient state of hyperadhesiveness of endothelial cells for neutrophils, but the molecular mechanisms underlying the preferential immigration of neutrophils into the skin upon application of LTB_4 are not completely understood. In this study, the effect of LTB_4 on the expression of adhesion molecules E-selectin, P-selectin, ICAM-1 and VCAM by HDMEC was investigated. Therefore, the endothelial cells were incubated with LTB_4 for 5 minutes and the expression of adhesion molecules was determined by immunocytochemical staining. LTB_4 induced the expression of E-selectin within two hours after incubation, but had no effect on the expression of ICAM-1, VCAM or P-selectin. E-selectin expression declined after 18 hours. Studies as to whether this represents a direct or indirect effect of LTB_4 are currently in progress.

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EXPRESSION OF ß1 INTEGRIN ANTISENSE RNA RESULTS IN REDUCED MIGRATION OF EPIDERMAL CELLS TO COLLAGEN TYPE I. T.S. Lange (1), K. Kirchberg (2), M. Wlaschek (2), Th. Ruzicka (1), Th. Krieg (2), K. Scharffetter-Kochanek (2), Departments of Dermatology, Heinrich-Heine-University Düsseldorf (1) and University of Cologne (2), FRG

Cell surface receptors of the ß1 integrin family serve as transmembrane linkers between the extracellular matrix and the cytoskeleton and promote cellular migration thus contributing to coordinated tissue repair. Quantitative regulation of integrin expression may at least in part be responsible for the fine tuning of the migratory response of epidermal cells after wounding. To approach this hypothesis we stably transfected a keratinocyte cell line (HaCaT) with a plasmid construct producing a ß1 integrin antisense RNA. Following PCR analysis and immunoprecipitation for the detection of antisense RNA and ß1 integrin synthesis, ß1 cell surface expression was monitored by FACS analyses. The effect of reduced ß1 integrin expression on directed cell migration to fibronectin or collagen type I was tested using the Boyden-chamber assay. Function blocking antibodies against different integrin subunits were introduced into the migration assay. All stably transfected cell clones synthesized ß1 integrin antisense RNA. In one cell clone synthesis and cell surface expression was downregulated by 50 %. This cell clone revealed a substantially reduced migratory response to collagen type I and fibronectin. Antibodies against defined integrin subunits confirmed that HaCaT cell migration to collagen type I is mediated by $\alpha_2\beta_1$ and to fibronectin by $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins. We show that the alteration of ß1 integrin biosynthesis and surface expression quantitatively regulates cell motility. Thus, modulation of ß1 integrin cell surface expression by antisense strategies, function blocking antibodies or growth factors might be of therapeutic value in wound healing disorders such as hypertrophic scar formation and keloids.

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ADHESIVE INTERACTIONS MEDIATED BY CD44 ISOFORM V10 (CD44R2)

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Expression of CD44 on lymphocytes has been implicated in hematopoiesis, lymphocyte recirculation into lymphatic tissue, and lymphocyte infiltration in cutaneous sites of delayed type of hypersensitivity. Recently, we have demonstrated that the CD44 splice variant consisting of exon v10 embedded into the CD44 standard framework (CD44R2) is expressed in malignant and reactive human skin lymphocytes. The objective of the present study was to determine whether CD44R2 may mediate cellular adhesive interactions that may be of functional relevance for lymphocyte infiltration into the skin. Therefore, a full-length CD44R2 cDNA obtained from a PCR-based CD44 expression library of microdissected tumor-stage mycosis fungoides was stably transfected into the CD44-negative human lymphoma cell line Namalwa. While untransfected parental cells exhibited a largely monodisperse distribution, CD44R2 transfectants spontaneously formed large homotypic cell aggregates. In antibody blocking experiments using CD44v10-specific mAbs VFF14 and VFF16, aggregation of transfectants could be inhibited confirming CD44R2-mediated homotypic cell-cell adhesion. Heterotypic cell-cell adhesion mediated by CD44R2 was analyzed by cell adhesion assays with human microvascular endothelial cells (HMEC) grown to subconfluency. Whereas unstimulated HMEC were not bound, stimulation with TNF-a and IL-1b for 6 hours induced strong binding of CD44R2 transfectants. In contrast, untransfected parental cells did neither bind to stimulated nor to unstimulated HMEC. Again, adhesion was blocked by preincubation of CD44R2 transfectants with CD44v10-specific mAbs. These results indicate that CD44R2 can promote homotypic as well as heterotypic cellular adhesive interactions that may be of functional relevance for the development of cutaneous lymphoid cell infiltration.

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SELECTIVE INHIBITORY EFFECTS OF RETINOIC ACID ON VCAM-1 GENE EXPRESSION IN MICROVASCULAR ENDOTHELIAL CELLS ARE MEDIATED THROUGH DIFFERENTIAL INHIBITION OF NF-κB-DEPENDENT BINDING TO CRITICAL NF-κB MOTIFS IN THE VCAM-1 PROMOTER. Jens Gille*, S. Wright Caughman, Thomas J. Lawley, and Robert A. Swerlick. Dept. of Dermatology, Emory University, Atlanta, GA, USA, and *Dept. of Dermatology, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany.

The expression of cell adhesion molecules (CAM) on endothelial cells is central to the pathogenesis of inflammatory processes. In order to determine modes of retinoid action in the inhibition of inflammatory responses, we have previously studied the effects of all-*trans* retinoic acid (t-RA) on the TNFα-induced expression of VCAM-1, ICAM-1, and E-selectin in dermal microvascular endothelial cells (HDMEC). Pretreatment with t-RA was found to specifically inhibit TNFα-induced VCAM-1 expression, but not ICAM-1 and E-selectin induction. Since this differential modulation of TNFα-induced CAM expression by t-RA was reflected at steady-state mRNA levels, we explored the molecular mechanisms involved in this differential inhibition of cytokine-mediated CAM induction by t-RA. In transcriptional activation studies, the TNFα-mediated activation of the human VCAM-1 promoter was inhibited after t-RA treatment, while the ICAM-1 promoter activation was unaffected, indicating that the selective inhibition of CAM expression is regulated in part at the level of gene transcription. Interestingly, the transcriptional inhibition by t-RA appears to be mediated by its effects upon the activation of NF-κB-dependent complex formation. Analysis of DNA-protein binding assays revealed marked inhibition of specific NF-κB-dependent binding to the tandem NF-κB sites of the VCAM-1 promoter, but not to the functional NF-κB motif of the ICAM-1 promoter. Since the NF-κB sites are necessary for both TNFα-induced VCAM-1 and ICAM-1 expression, these findings emphasize the functional significance of these motifs in t-RA-mediated inhibition of the regulated VCAM-1 gene transcription, and also provide a rational explanation for the lack of t-RA-mediated inhibition of ICAM-1 promoter activation. This work thus further delineates the selective transcriptional inhibition within the context of different NF-κB-regulated promoters (VCAM-1 vs. ICAM-1), and demonstrates for the first time selective inhibition of retinoids upon NF-κB-dependent gene expression.

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IMMUNOPHENOTYPIC CHARACTERISTICS OF DENDRITIC CELLS DETERMINE THEIR BIOLOGIC FUNCTION IN VIVO

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The potential use of dendritic cells (DC) for immunotherapy requires a detailed study of their biologic function *in vivo*. For this purpose we compared DC propagated from various sources under different culture conditions in their capacity to elicit primary immune responses *in vivo*. The following DC types were obtained from C3H ($H\text{-}2^k$) mice and were employed in an allogeneic skin transplantation model: i) DC from bone marrow (BM)-cultures propagated in GM-CSF (BMDC-GM, MHC class $II^+/CD80^{dim}/CD86^{dim}$), ii) DC from BM-cultures in the presence of GM-CSF and IL-4 (BMDC-GM4, MHC class $II^+/CD80^+/CD86^+$), iii) a fetal skin-derived DC line (80/1, MHC class $II^+/CD80^+/CD86^+$). Using BALB/c ($H\text{-}2^d$) mice as recipients, a C3H allograft was rejected with a mean survival time (MST) of 10.7 d (first set). Single injections (s.c.) of 10^6 BMDC obtained from both culture conditions sensitized H-2-disparate recipients for specific transplantation immunity as evidenced by significantly accelerated rejection of skin allografts from C3H mice, but not of third-party allografts. However, BMDC-GM4 injected s.c. were more effective in their sensitizing capacity than the same number of BMDC-GM (BMDC-GM4: MST 7.7 d, in comparison to first set rejections $p<0.05$; BMDC-GM: MST 8.3 d, $p<0.05$). Differences became more obvious by immunization via the i.v. route (BMDC-GM4: MST 8.4 d, $p<0.05$; BMDC-GM: MST 9.0 d, NS). Titration experiments with BMDC-GM4 revealed that as few as 10^4 cells, administered by the s.c. route, were effective. When compared with the skin-derived 80/1 DC, which have previously been demonstrated to sensitize effectively *in vivo*, 10^4 cells of both cell types induced a similar MST (80/1 DC: MST 9.0 d, $p<0.01$; BMDC-GM4: MST 8.8 d, $p<0.01$).)

We conclude that mature DC obtained from murine skin and BM are capable of eliciting primary immune responses *in vivo*, and that the propagation of DC from various sources under different culture conditions can lead to phenotypically and functionally distinct DC populations. These distinct populations may be useful tools for either immunizing or tolerizing strategies.

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THE DISEASE SPECIFIC PHENOTYPE OF HUMAN LANGERHANS CELLS AND INFLAMMATORY DENDRITIC EPIDERMAL CELLS IS OF DIAGNOSTIC VALUE IN CUTANEOUS INFLAMMATION.

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In normal human skin, the homogenous population of Langerhans cells represents the family of antigen presenting dendritic cells. In contrast, eczematous skin harbours two distinct epidermal dendritic cell populations: $Fc\varepsilon RI^+$, $CD1a^{++}$, Birbeck granule positive 'classical' Langerhans cells (LC) and $Fc\varepsilon RI^{++}$, $CD1a^+$, Birbeck granule negative Inflammatory Dendritic Epidermal Cells (IDEC). Skin biopsies (n=229) from psoriasis, atopic eczema, mycosis fungoiodes, allergic contact eczema and other inflammatory skin diseases were investigated by quantitative flow cytometry for evaluation of Fc-receptors, adhesion molecules and other surface molecule expression patterns. LC and IDEC were present in variable percentages in almost all inflammatory skin samples. Regarding adhesion molecules, E-, L- and P-Selectin expression was not detected on either cell population. IDEC were, in contrast to LC, strongly reactive for the integrin chains CD11a and CD11b in all diseases studied, representing the the most reliable marker for their distinction on a phenotypic level. In contrast to IDEC, consistently expressing the VLA4 α-chain, this molecule was upregulated on LC in psoriasis. IDEC expressed all $Fc\varepsilon Rs$ and $Fc\gamma Rs$, showing a significant ($p\leq0{,}05$) disease specific upregulation for $Fc\varepsilon RI$ in atopic eczema and for $Fc\gamma RII$ and $Fc\varepsilon RII$ in psoriasis vulgaris. An expression ratio of $Fc\varepsilon RI/Fc\gamma RII$ allowed differential diagnosis of atopic eczema from all other skin diseases including allergic contact eczema with 100% sensitivity and 94% specificity. In conclusion, surface receptor profiles of LC and IDEC are influenced by the epidermal micromilieu in a disease specific manner, representing a useful tool for basic research and differential diagnosis of cutaneous inflammation.

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FK506 (TACROLIMUS) IMPAIRS THE PHENOTYPIC AND FUNCTIONAL DIFFERENTIATION OF HUMAN EPIDERMAL LANGERHANS CELLS

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The immunosuppressive drugs ciclosporin A and FK506 are used in the management of chronic inflammatory skin diseases like psoriasis and atopic dermatitis. Although these drugs are known to mainly act at the level of T cells, other immunocompetent cells may also be considered as relevant targets. Since it has been shown recently that topical FK506 is effective in the treatment of atopic dermatitis where a pathophysiological role of epidermal Langerhans cells (LC) has been suspected, we explored the *in vitro* effect of this compound on LC. In a first approach, LC were freshly isolated from normal skin and cultured in the presence of GM-CSF and increasing concentrations of FK506 (10^{-9} to 10^{-6} M). Flow cytometric assessment of viability by 7AAD staining at 18h and 36h culture did not show any significant effect on keratinocytes or LC at these concentrations. Then double labeling experiments show that FK506 inhibited the appearance/increase of distinct functionally relevant molecules like HLA-DR and most importantly B7.1 and B7.2. In order to investigate whether these phenomenons have any functional relevance, the stimulatory capacity of normal and FK506-treated LC was checked in SMLR experiments. Hereby, a dose dependent decrease in the stimulation could be noted in LC cultured in the presence of FK506. Thus, we provide for the first time evidence that FK506 impairs the biology of human LC and may explain, at least in part, the therapeutical effect of this drug in eczematous skin diseases.

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DIFFERENTIAL GENE EXPRESSION IN EPIDERMAL LANGERHANS CELLS MONITORED BY DIFFERENTIAL DISPLAY, R. Roß, A. Endlich, K. Kumpf, J. Schwing and A. B. Reske-Kunz, Clinical Research Unit, Department of Dermatology, Johannes Gutenberg University, Mainz, Germany

Following short-term culture, Langerhans cells (LC) mature morphologically and functionally into potent immunostimulatory cells. Differential gene expression accompanies this maturation process and it is likely that these differentially expressed genes are involved in the maturation events.

We used the recently described method of differential display to identify genes differentially expressed in murine epidermal LC isolated either directly (fLC) or following three day cultivation (cLC). Cloning and sequencing of the isolated cDNA fragments, followed by database searches revealed that so far unknown genes as well as already characterized genes were identified.

The activation marker 4F2, belonging to the group of the latter genes, was shown before to be highly expressed on tumor cells and to be upregulated during cell division on a wide range of cell types. We provide evidence that 4F2 is downregulated on cLC at the mRNA and protein level, indicating that the committed, terminally differentiated cLC downregulate proteins involved in proliferation and cell division.

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CD33 POSITIVE MYELOID PRECURSOR CELLS ISOLATED FROM HUMAN DERMIS DEVELOP INTO POTENT DENDRITIC ANTIGEN-PRESENTING CELLS. Frank O. Nestle, Florence Raboud, Beatrix Müller, and Günter Burg, Department of Dermatology, University of Zürich Medical School, Zürich, Switzerland.

Recently, a population of dendritic antigen-presenting cells (APCs) has been identified in dermis of normal human skin. The purpose of the present study was to define a putative precursor population of these cells. In-vivo studies demonstrated CD33 positive cells mostly in a perivascular localization in the upper dermis. Fresh cell suspensions of human dermis were incubated with a moAb to CD33, which is expressed on cells of the myelomonocytic lineage. The respective cell population was isolated with high gradient magnetic cell separation (MACS) and placed in culture in the presence or absence of GM-CSF and IL-4. Human dermal CD 33 positive cells were >95% pure and co-expressed high levels of CD13. They were non-adherent to plastic and displayed a round cell morphology. After a culture period of 6-7 days, cells were still non-adherent to plastic and started to develop a dendritic morphology which was more pronounced in the presence of GM-CSF and IL-4. During the maturation step in culture a marked increase in the expression of HLA-DR as well as co-stimulatory molecules such as CD86 (B7-2) was observed. Subpopulations of cells expressed CD14 or CD1a, which has also been shown in dendritic cells generated from CD34 positive bone marrow precursor cells. All cells expressed CD1c and were positive for the moAb 3.29 which recognizes dendritic cells of the dermis but not epidermis. Functional studies using various mitogens and autologous CD4 T cells as responders demonstrated similar stimulatory capacities of CD33 negative and CD33 positive cells. After culture a marked and comparable increase in the stimulatory capacity of CD33 positiv but not CD33 negative cells was observed. Therefore, defined by their morphology, phenotype and function, CD33 positive resident cells of human dermis acquire characteristics of dendritic cells in culture. The presence of resident dendritic cell precursors in human dermis suggests a role for these cells in skin immune responses.

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INTERCELLULAR ADHESION IN AGGREGATES OF MURINE FETAL SKIN-DERIVED DENDRITIC CELL PRECURSORS IS MEDIATED BY E-CADHERIN
Thilo Jakob and Mark C. Udey, Dermatology Branch, NCI, NIH, Bethesda, MD USA

Cadherins mediate calcium-dependent homophilic adhesion between various epidermal cells, including adhesion between Langerhans cells (LC) and keratinocytes. To study the regulation LC-keratinocyte adhesion, we propagated LC-like dendritic cells from day 16 C57BL/6 fetal skin in GM-CSF and CSF-1-supplemented media. After 2 wks, E-cadherin+ (E-Cad) fetal skin derived dendritic cells (FSDC) that resembled cultured LC in terms of morphology, phenotype and function were obtained. FSDC represent nonadherent single cells that appeared to develop from clusters of tightly aggregated precursors. Immunoprecipitation and western blotting studies using fetal skin-derived leukocytes demonstrated that E-Cad was noncovalently bound to cytoplasmic proteins (α-, β-, and γ-catenin and $p120^{Cas}$) which are known to link E-Cad to the cytoskeleton and which are required for high affinity adhesion. Immunofluorescent staining of aggregates of permeabilized FSDC precursors revealed concentration of E-Cad, β- and γ-catenin, and $p120^{Cas}$ in areas of cell-cell contact, suggesting that intercellular adhesion might be E-Cad-mediated. Aggregates were resistant to dissociation by trypsin in presence of Ca^{++}, but were dissociated by trypsin in absence of Ca^{++}. Furthermore, addition of E-Cad-specific mAb ECCD-1 and DECMA-1, which inhibit E-Cad function, also dissociated aggregates within 18 h, whereas equal amounts of E-Cad-specific mAb ECCD-2 (that does not interfere with function), anti-ICAM-1 mAb (3E2), and appropriate isotype controls did not. Monoclonal Ab reactive with Mac-1 (M1/70), LFA-1 (M1/17), ICAM-1 (YN1/1.7.4) and CD45 (M1/9.3.4.) also stained cells within aggregates, but did not interfere with adhesion. We conclude that intercellular adhesion within aggregates of FSDC precursors is primarily mediated by E-Cad. We anticipate that further studies of FSDC precursors will provide insight into E-Cad-mediated adhesion involving LC.

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IL-4 ENHANCES THE YIELD AND THE IMMUNOSTIMULATORY POTENCY OF MURINE BONE MARROW-DERIVED DENDRITIC CELLS.
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Dendritic cells (DC) far excel other cell populations in the capacity to stimulate naive T cells. Recently, Inaba and colleagues reported the successful generation of large numbers of murine DC from bone marrow (BM) under the aegis of GM-CSF. Because IL-4 is known to modulate DC function, we added IL-4 to the BM-cultures and investigated the effect of this cytokine on the phenotype and function of the resulting DC. Lymphocyte-depleted BM cells were cultured in medium supplemented with GM-CSF or GM-CSF and IL-4 for 6 days. At this time, loosely- and non-adherent cells were harvested, subcultured for another day and then analyzed. 8-33% ($3\text{-}6\times10^5$ cells/mouse; n=16) of the cells grown in GM-CSF and 42-63% ($2\text{-}4\times10^6$ cells/mouse; n=14) of the cells cultured in GM-CSF+IL-4 were identified as DC on the basis of their distinctive morphology and their antigen expression pattern (CD45, MHC class II^{bright}, NLDC145, N418, CD25, *rel*/B) as determined by flow cytometrical analysis. Expression of costimulatory molecules (B7-1, B7-2, ICAM-1, HSA) was more pronounced on DC generated in GM-CSF+IL-4 (BMDC-GM4) compared to cells cultured in GM-CSF alone (BMDC-GM). To assess their immunostimulatory capacity we FACS-purified MHC class II^{bright} cells from BMDC-GM/GM4 bulk cultures and used them to stimulate naive, allogeneic T cells. BMDC-GM4 were 3-4x more potent stimulators of the primary MLR than BMDC-GM. The contact hypersensitivity system and a model for transplantation immunity were used to test the capacity of these BMDC to elicit primary immune responses *in vivo*. In both models BMDC-GM4 induced a stronger immune response than BMDC-GM.

In summary, culture of BM cells in medium supplemented with GM-CSF leads to the generation of mature DC and the addition of IL-4 to the cultures dramatically increases the yield of DC as well as their immunostimulatory capacity. This approach to generate high numbers of potent DC may prove useful for vaccination studies.

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FUNCTIONAL ROLE OF CDw101 FOR T LYMPHOCYTE ACTIVATION BY SKIN DENDRITIC CELLS. Isabelle Martinel, Dominique Charue, Marie-Laure Boulland*, Janine Wechsler*, Laurence Boumsell, Armand Bensussan, and Martine Bagot. INSERM U 448 and *Department of Pathology, Paris XII University, Créteil, France.

CDw101 is a type I trans-membrane protein with seven Ig-like loops in its extracellular domain, that has a molecular weight of about 140 kDa. It was first described in our laboratory by three differents monoclonal antibodies (mAb), BB27, BA27 and BC27, recognizing CDw101 as a disulfide bonded-homodimer on subsets of circulating T lymphocytes (20-25%). We also found that it is expressed on most activated T cells *in vitro*. The gene coding for the CDw101 antigen has been cloned and found to be identical to the gene coding for the recently described V7 antigen, recognized by the V7-1 mAb. However, cross-block experiments show that BB27 and V7-1 recognize different epitopes of CDw101. We studied the expression of CDw101 on skin dendritic cells (DC) migrating from human skin explants, and the effect of this molecule on mixed epidermal cell-lymphocyte reaction. Phenotypic studies showed that migrating DC expressed HLA-DR (85%), CD1a (82%), CD1c (65%), CD86 (64%), CD45RO (60%), ICAM-3 (75%), ICAM-1 (77%), LFA-1 (87%), LFA-3 (72%) and CDw101 (29-79%). After an epidermal-dermal separation by dispase, we observed that dermal dendritic cells expressed CDw101 (40%) more than the epidermal DC (20%). This result was confirmed by *in situ* immunophenotyping. Simple and double labeling showed a large number of CDw101-positive DC, more numerous in the dermis than in the epidermis. Only a part of these expressed CD1a. Functionally, CDw101 seemed to play an important role in T cell activation by DC, since an anti-CDw101 monoclonal antibody specifically inhibited a mixed epidermal cell-lymphocyte reaction (65%). Preincubation assays showed an inhibitory effect both on T cells and on DC. In conclusion, CDw101 which is expressed on a subpopulation of circulating T lymphocytes, has also been found on a sub-population of DC particularly in the dermis. In addition, we have shown that this molecule plays a major role in the activation of T cells by skin DC.

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THE RELATIVE CONTRIBUTION OF DIRECT VERSUS INDIRECT PRESENTATION IN PRIMING FOR CONTACT HYPERSENSITIVITY BY MHC CLASS I^+/CLASS II^- DENDRITIC CELLS.
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Dendritic cells (DC) have been proposed as suitable tools for immunizing strategies *in vivo*. We have recently demonstrated that a unique $CD45^+$/MHC class $I^+(H\text{-}2^k)$/class II^-/$CD80^+$ DC line (80/1), when derivatized with the hapten trinitrophenyl (TNP), can induce hapten-specific MHC class I-restricted responses *in vitro* and contact hypersensitivity responses (CHS) *in vivo*. To elucidate the mode of antigen presentation occurring in this system, we compared the sensitizing capacities of identical numbers (10^6, injected s.c.) of viable and dead haptenized 80/1 cells and L929 ($H\text{-}2^k$) fibroblasts. The only cells capable of initiating a CHS response were live TNP-derivatized 80/1 cells, implying that 80/1 DC, but not fibroblasts, can migrate to the regional lymphoid organs and elicit a hapten-specific response in naive, $CD8^+$ T cells. Surprisingly, when we examined the immunophenotype of lymphocytes infiltrating CHS lesions in animals primed with TNP-derivatized 80/1 cells, we observed that at the peak of the response the major proportion of T cells is of the $CD4^+$ rather than the $CD8^+$ phenotype. In the next series of experiments, we asked whether *in vivo* depletion of either T cell subset interferes with the induction of 80/1 cell-induced CHS response. Mice received 3 daily injections of 0.4 mg of either anti-CD4 or anti-CD8 antibody prior to immunization, and one additional injection of antibody the day before challenge with trinitrochlorobenzene. Results obtained showed that elimination of either $CD4^+$ or $CD8^+$ T cell subset led to a significant reduction in the ear swelling response as compared to animals that had received isotype control antibody.

Based on these findings we suggest two pathways of hapten presentation by 80/1 DC, i.e. direct activation of $CD8^+$ T cells in an MHC class I-restricted fashion and, in addition, uptake and presentation of TNP-derivatized 80/1 cell fragments by host APC using the MHC class II pathway.

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UPTAKE AND INTRACELLULAR ROUTE OF THE MANNOSE RECEPTOR AND MANNOSYLATED ANTIGENS IN HUMAN DENDRITIC CELLS. Mieke Mommaas, Agnes Tan, Jos Onderwater, Aat Mulder, Reina Jordens, Annette van der Heiden, Desiree Verwoerd, Antonio Lanzavecchia, Jacques Neefjes, Abraham Tulp, and Frits Koning, Depts of Immunohematology and Blood Bank and Dermatology, and Lab for Electron Microscopy, University Hospital Leiden, Division of Cellular Biochemistry, Dutch Cancer Institute Amsterdam, The Netherlands, Basel Institute for Immunology, Basel, Switserland.

Dendritic cells (DC) are unique in their potency to stimulate antigen-specific responses in vitro and in vivo. In an immature stage, they are extremely efficient in antigen uptake as it is mediated by various endocytic pathways including macropinocytosis and clathrin-mediated internalization. The unique expression of a mannose receptor on DC, certain epithelial cells, and macrophages contributes to the selectivity and efficiency of uptake of mannosylated antigens which are usually of non-self origin. Therefore, mannosylation of an antigen of interest might be a useful tool to induce its selective uptake and presentation by DC. For this purpose, we have determined the route and kinetics of intracellular transport of the mannose receptor, and mannosylated and non-mannosylated antigens in DC by means of Subcellular Fractionation using a Density Gradient Electrophoresis Device and Immunoelectron Microscopical analysis. We found that both the kinetics and the route of mannosylated antigens are distinct from that of non-mannosylated antigens. Uptake of mannosylated antigens occurs much more rapidly and the endocytic route taken by mannosylated and non-mannosylated antigens do not overlap initially. Moreover, the observation that the mannose receptor and MHC class II molecules do not co-localize suggests that the mannose receptor, which recycles constitutively, releases its ligand and recycles back to the plasmamembrane from a compartment preceding the MIIC. In addition, our results suggest that MHC class II molecules passage through the MIIC very quickly.

Thus, our data indicate that the mannose receptor on DC mediates efficient uptake of mannosylated antigens by an endocytic route that is clearly distinct from (macro)pinocytosis. Currently, we investigate whether this results in a more efficient presentation of peptides derived from mannosylated (tumor) antigens compared to its non-mannosylated counterparts.

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INCREASED EXPRESSION OF MHC CLASS II MOLECULES ON HUMAN MONOCYTES INDUCED BY SUBPHYSIOLOGICAL TEMPERATURES. A. Felli, IM. Schmitt, S. Chimenti. Department of Dermatology, University of L'Aquila, Italy.

MHC class I & II molecules, membrane glycoproteins known to be essential for antigen recognition and immunogenicity allow the immune system to discriminate between self and non-self antigens. In this study we evaluated the effects of physiological and subphysiological temperatures (37°, 26°C) on MHC class I and II molecule expression on the human immunocompetent lymphocytes and monocytes. Peripheral blood mononuclear cells (PBMC) from healthy volunteers were isolated by Fycoll-Hypaque gradient sedimentation. FACS analysis was performed using antibodies FITC-labeled specific for human MHC class I and II molecules (HLA A,B,C, and HLA,-DP,-DQ, DR respectively). Lymphocytes kept *in vitro* in culture at 26°C did not show any substantial increase of HLA A,B,C expression on the cell surface after several hours of cell colture (24,48,72) in comparison to time point O, whereas monocytes kept for a 48 hr period under identical culture conditions showed a 22% increase in comparison to "freshly" isolated monocytes (control). Furthermore, a 4-5 fold increase of MHC class II molecules on monocytes incubated at 26° C in comparison to the control was observed. This increase was seen after 24 hrs of incubation with a plateau at 48 hrs. Monocytes incubated at 37° C also presented, after 24-48 hrs, a 2-fold increase in class II molecules in comparison to the control. In addition, frozen monocytes incubated at 37° C for 48 hrs showed a dramatic up-regulation of class II (10-11 fold). We hypothesize that stress factors (subphysiological temperatures or PUVA) could modify the antigen presenting cell function of human monocytes.

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ISOLATION OF EPIDERMAL PEPTIDE ANTIGENS FOLLOWING LARGE-SCALE CULTIVATION OF KERATINOCYTES. D Dressel[1,2], H Max[2], H Kalbacher[2], HE Meyer[3], W Sterry[4], W-H Boehncke[1], Universities of Ulm[1], Tübingen[2], Bochum[3], Berlin/Charité[4], Germany.

Antigens relevant for organ-specific autoimmune diseases might be identified by comparing HLA-bound peptides in a compartment-depending manner. A prerequisit for this is the generation of the respective antigen presenting cells in sufficient numbers and purity. We here describe a technique allowing to grow large numbers of human keratinocytes in the absence of feeder cells. Punch biopsies were trypsinized over night and transfered into KGM medium (Promocell, Heidelberg) allowing selective growth of keratinocytes. Following an initial culture period of 14 days cells were split 1:3 every 5-6 days and finally stimulated with 10^3 U/ml IFN-gamma resulting in expression of HLA class II molecules. The harvest was 10^9 cells after 10 passages. Purification of HLA molecules from lysed cells by affinity chromatography yielded 300µg HLA-DR and 170µg HLA-DQ. HLA bound peptides were released at pH 2.0 and further analysed by microbore reverse phase HPLC followed by matrix assisted laser desorption ionisation mass spectroscopy. This approach allowed identification of several prominent peaks of 1-1.6kD which were identical in material from both patients analysed so far. This technique is thus suitable for generation of large numbers of pure keratinocytes. Initial observations suggest that candidate peptides can be identified for HLA class II restricted T cell responses to epidermal targets.

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ERYTHROMYCIN RESISTANT PROPIONIBACTERIA: LABORATORY MUTANTS AND CLINICAL ISOLATES FROM ACNE PATIENTS ARE PHENOTYPICALLY SIMILAR. E. Anne Eady, Yvonne W. Miller, Sandhya Vyakrnam, Hamid Ratyal, Jonathan H. Cove, and *William J. Cunliffe, The Skin Research Centre, Dept. of Microbiology, Univ. of Leeds and *Dept. of Dermatology, Leeds General Infirmary, Leeds, U.K.

One in three antibiotic treated acne patients carry erythromycin resistant propionibacteria. Carriage of resistant strains is associated with failure to respond to oral erythromycin therapy. The mechanism by which resistance has been acquired is unknown. Erythromycin resistant mutants were selected by plating two laboratory strains of fully sensitive propionibacteria (one *P. acnes* and one *P. granulosum)* onto agar containing 5 µg/ml of the drug (equivalent to 100 × MIC). In order to rule out the possibility that the erythromycin resistant variants were contaminants rather than mutants, the experiment was repeated with doubly marked derivatives of each sensitive parental strain. Resistance patterns of the mutants were compared with those of clinical isolates from acne patients and were found to be either similar or identical in that they were cross resistant to 14- and 16-membered ring macrolides, lincosamides and type B streptogramins. Only the degree of resistance varied between isolates. This observation suggests that erythromycin resistant propionibacteria may have arisen by mutation within the gene encoding 23S rRNA (part of which contributes to the erythromycin binding site in 50S ribosomal subunits) as has previously been shown for *Mycobacterium intracellulare* and *Helicobacter pylori*.

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T-LYMPHOCYTE RESPONSES TO HEAT SHOCK PROTEIN 70 IN PATIENTS WITH ACNE VULGARIS AND NORMAL CONTROLS Andrew M.Fruin, H.Ruth Ashbee, Keith.T.Holland, William J.Cunliffe* and Eileen Ingham. The Skin Research Centre, Departments of Microbiology and Dermatology*, University of Leeds, Leeds, UK.

The role of *P.acnes* in inflammatory acne vulgaris cannot be explained by the numbers or strains colonising affected follicles. It is possible that in some follicles, *P.acnes* is stressed and expresses immunogenic heat shock proteins (hsp) which may play a role in the T-cell mediated inflammatory response of acne lesions. The T-cell response to *P.acnes* and hsp 70 (*Mycobacterium smegmatis*) of 10 acne patients and 10 age and sex matched controls was compared using lymphocyte transformation assays (TI) and leucocyte migration inhibition (MI). There was no significant difference in the TI to *P.acnes* of patients (mean TI = 2.34) or controls (1.41) or the TI to hsp 70 of patients (2.00) or controls (1.07). However, 5/10 patients gave a positive TI (> 2) to hsp 70 compared to one control. There was no significant difference in the MI to hsp 70 of patients (mean MI = 0.85) or controls (0.96). Patients had a significantly different MI (0.5) than controls (0.65) to *P.acnes* ($p<0.05$). Again 50% of patients gave a significant MI (<0.8) to hsp 70 compared to one control. Thus, the trend towards hsp 70 reactivity in acne patients indicates that further studies on *P.acnes* hsp are warranted.

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PHOTOTHERAPY OF ACNE VULGARIS WITH VISIBLE LIGHT

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Sun exposure has a beneficial effect on acne vulgaris. However, until now it's not clear which wavelengths contribute to the favourable effect. We studied the effect of ultraviolet (UV) light, visible light and the combination of both. We were especially interested in the effect of visible light, since UV exposure is known to be related to an increased incidence of skin cancer. Thirty patients (15 men and 15 women) with mild to moderate acne vulgaris, involving the face and/or the back and/or the chest, were treated with three different light sources, three times weekly, for seven weeks.

All three light sources used: "full spectrum" (emitting visible light and UV-A), green and violet light improved the acne, leading to 14% ($p>0.10$), 22% ($p<0.05$) and 30% ($p<0.02$) improvement, respectively. No statistically significant differences between the light sources were found, although violet light apperared somewhat better than the others. No side-effects were observed.

Visible light is an effective and safe alternative for treatment of acne vulgaris. Since both violet and green light are capable of activating porphyrins, that are produced by P. acnes, we speculate that phototherapy acts mainly on P. acnes probably by causing photodynamic destruction.

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PRECOMEDONAL EVENTS IN ACNE. Odette L. Aldana, Diana B. Holland, and William J. Cunliffe, Dept of Dermatology, Leeds General Infirmary, Leeds, U.K.

Many aspects of acne remain inadequately explained. The precise mechanism by which ductal cornification leads to comedone formation is uncertain. We investigated proliferation in apparently normal follicles biopsied from acne patients using the monoclonal antibodies K16, a marker for hyperproliferation and Ki-67, a marker of cycling cells. K16 labelling was present suprabasally and identified in one of three locations: location A- sebaceous gland/follicle junction; location B- sebaceous gland and follicular duct; location C - sebaceous gland, follicular duct and interfollicular epidermis. Ki-67 positive cells were located in the basal layer of the epidermis. Positive nuclei were counted in the follicle wall, perifollicular and interfollicular epidermis, and then expressed as a percentage of the total number of nuclei in each area. Results from these analyses fell into 4 distinct groups: group 1- low percentage Ki-67 and K16 in locations A and B; group 2- high percentage Ki-67 and K16 in locations A and B; group 3- high percentage Ki-67 and K16 in locations B and C; group 4- low percentage Ki-67 and K16 in locations B and C. At the same time histological observations on some follicles showed microcomedonal features eg. distended duct and thin follicular walls particularly in groups 3 and 4. Thus in acne many clinically normal follicles are not normal. They are subclinically undergoing hyperproliferation and may present as 1 of 4 distinct dysfunctional states which correspond to different stages of a follicular cycle. Dependent on local environmental cues these follicles can progress to comedogenesis or resolution. Studies on comedone formation must take these observations into account.

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PROLIFERATION AND DIFFERENTIATION OF CULTURED NORMAL HUMAN KERATINOCYTES ARE MODULATED BY TREATMENT WITH MINOCYCLIN. G. Meneguzzi, O. Partouche, [1], J.-P. Ortonne. INSERM U385, UFR de Médecine, Avenue de Valombrose, Nice, [1]Lederle Laboratories, 74 rue d'Arcueil, Rungis, France.

The abnormal keratinization of the infra-infundibular zone of the follicular epithelium seems to play an important role in the pathogenesis of acne vulgaris. Clinical studies have shown that the treatment with minocyclin correlates with a decrease in the number of the retentional lesions. This observation cannot be explained by the antibacterial and/or anti inflammatory effect of the treatment. We have therefore evaluated the effect of minocyclin on proliferation, keratinization and terminal differentiation of human normal keratinocytes (HNK) in vitro.

Secondary cultures of HNK, grown on mouse 3T3 feeders and in the presence of high calcium levels, were exposed to increasing concentrations of minocyclin. The growth rate of the treated cell cultures, determined after trypsinization and numeration of the living keratinocytes, revealed that at a concentration of 6 µg/ml the antibiotic exerts an inhibitory effect on cell proliferation. Western analysis of total cellular extracts at days 5 and 12 of the treatment was then performed using antibodies raised against keratins K14, K10, filaggrin, loricrin and involucrin. The expression pattern of these proteins in the presence of minocyclin suggested an inhibition of cell differentiation notably at the terminal stages of the process.

Concordant with the results of previous immunohistological analyses performed on skin biopsies of patients presenting with acne, our results indicate that besides the bactericidal effect, minocyclin might exert an inhibitory action on terminal differentiation of the keratinocytes of the infundibular epithelium. Studies on the possible role of this antibiotic on the regulation of transcription of the genes involved in the terminal differentiation of the keratinocyte are in progress.

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AUTONOMIC INNERVATION OF MURINE SKIN: HAIR CYCLE-DEPENDENT REMODELING AND HAIR GROWTH-INDUCTION BY DRUGS MODULATING ADRENERGIC FUNCTION. E.M.J. Peters, M. Maurer, V.A. Botchkarev, S. Eichmüller, and R. Paus. *Dept. of Dermatology, Virchow Hospital, Humboldt-Universität zu Berlin, D-13353 Berlin, Germany.*

Keratinocytes express receptors for cholinergic and adrenergic agents and the autonomic nervous system has been suggested to be involved in the control of epithelial tissue growth and remodeling (JID 104: 953, JID 98: 457). In order to further explore previously underappreciated functions of the autonomic nervous system in skin physiology, we have studied the autonomic innervation of C57BL/6-mouse skin during various stages of the depilation induced hair cycle. In addition, the functional effects of two noradrenaline (NA)-depleting agents on hair follicle cycling in these mice were investigated. Applying immunocytochemistry and the paraformaldehyde or glyoxylic acid condensation method, the number of tyrosine-hydroxylase-immunoreactive (TH-IR) and NA-containing fibers as well as the number of cholineacetyl-transferase-immunoreactive (ChAT-IR) nerve fibers was found to be increased in the growing phase of the hair follicle (anagen), followed by a significant decrease of TH-IR and NA-containing fibers towards catagen. The NA-depleting agents 6-OHDA or guanethidine injected s.c. to the shaven lower back skin of 24 test and 14 vehicle telogen C57BL/6-mice (6-OHDA: 200µl, 0.03 mg/g body weight [bw], days 0 and 1; guanethidine: 200µl, 0.02 mg/g bw on day 0, 0.04 mg/g bw on day 3) induced the onset of anagen. On day 21 after the start of treatment, more than 80% of the guanethidine-treated mice and ca. 65% of the 6-OHDA-treated mice exhibited anagen at the site of application. Only less than one third of all control animals showed macroscopic signs of anagen development, and here the anagen region was smaller than in test mice and likely reflected traumatic anagen induction by injection. These macroscopic observations were confirmed by histology, demonstrating mature anagen VI follicles only at the immediate site of treatment with 6-OHDA or guanethidine as opposed to resting telogen follicles in the neighbouring untreated skin area. These observations support the concept that hair cycling in mice may not only be modulated by neuropeptides released from sensory nerves in the skin (cf. Lab Invest 1994: 134), but also by neurotransmitters released from cutaneous nerve fibers of the autonomic nervous system.

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TOPOBIOLOGY OF THE HAIR FOLLICLE: I. EXPRESSION OF CELL-ADHESION MOLECULES DURING NEONATAL HAIR FOLLICLE MORPHOGENESIS IN MICE Sven Müller-Röver, Yoshiki Tokura* and Ralf Paus Depts. of Dermatology, Virchow-Hospital, Humboldt Universität zu Berlin, D-13353 Berlin, Germany and *Hamamatsu University School of Medicine, Hamamatsu, Japan.

The postnatal development of hair follicles is brought about by bidirectional interactions between epidermal and mesenchymal tissues. The molecular basis of these interactions is still enigmatic. Since cell-adhesion molecules (CAMs) may play a pivotal role during this process, we have studied the immunohistological expression pattern of selected CAMs during neonatal murine hair follicle development (C57BL/6): neural cell adhesion molecule (NCAM), E- and P-cadherin (Ecad; Pcad), intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen (LFA-1) and macrophage differentiation antigen (MAC-1). During the initial stages of hair follicle development, the strong dermal NCAM immunoreactivity (IR) became restricted to the fibroblasts of the future dermal papilla and of the perifollicular connective tissue sheath (pCTS). Ecad IR was initially present on all epithelial cells and was progressively downregulated on the developing inner root sheath (IRS) and the inner layers of the hair matrix (IHM). Throughout hair follicle development, the basal epidermal layer showed slight Pcad IR. During epidermal invagination, all downgrowing follicle KCs of the hair peg were $Pcad^+$. Consecutively, the developing IRS and the sebocytes showed decreased Pcad IR. At the same time, the outer root sheath (ORS) and - in contrast to Ecad - the IHM showed increasing Pcad IR. The first weak follicular ICAM-1 expression was found in the periinfundibular region of the ORS (piORS) in fully developed anagen VI hair follicles and in the proximal CTS during the first postnatal catagen. Only scattered $LFA\text{-}1^+$ cells were found homogenously distributed in the interfollicular epidermis and dermis. Increasing numbers of $LFA\text{-}1^+$ cells were concentrated around the piORS. An increasing number of $MAC\text{-}1^+$ cells was restricted mainly to the perifollicular subcutis. Our results suggest that ICAM-1, LFA-1 and MAC-1 may be more important for the first catagen-associated follicle remodeling than for hair follicle neogenesis. Instead, NCAM expression may be a crucial event in the condensation of the dermal papilla and the pCTS. $Pcad^+$ KCs of the basal layer of neonatal epidermis may represent a defined subpopulation of epidermal KCs destined to grow down and form the new hair peg.

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TOPOBIOLOGY OF THE HAIR FOLLICLE: II. EXPRESSION OF CELL-ADHESION MOLECULES DURING HAIR FOLLICLE CYCLING P. Welker, S. Müller-Röver, Y. Tokura*, V. Botchkarev and R. Paus Deps. of Dermatology, Virchow-Hospital, Humboldt Universität zu Berlin, Germany and *Hamamatsu University School of Medicine, Hamamatsu, Japan.

Cell-adhesion molecules (CAMs) may be involved in the induction of hair follicle growth (anagen) and regression (catagen). To further elucidate the role of CAMs in mature hair follicle cycling, we have correlated the distinct spatiotemporal expression patterns of selected CAMs with defined stages of the depilation-induced hair cycle in C57BL/6 mice, studying the immunohistological expression of neural cell adhesion molecule (NCAM), E- and P-cadherin (Ecad; Pcad), intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen (LFA-1) and macrophage differentiation antigen (MAC-1). NCAM immunoreactivity (IR) was mainly restricted to dermal papilla (DP) fibroblasts and the perifollicular connective tissue sheath (CTS). After anagen induction there was a substantial upregulation of NCAM expression on DP fibroblasts (and, weaker, on the CTS) which decreased again during early catagen. During telogen Ecad IR was found on all epithelial cells, including the cells of the bulb and the sebaceous gland. Pcad IR, instead, was strikingly restricted to a subpopulation of epithelial cells of the telogen germ region, directly overlying the DP. During anagen the inner layers of the proliferating hair matrix showed strong Pcad IR and no Ecad IR at all. During hair follicle cycling the majority of $LFA\text{-}1^+$ cells was concentrated around the $ICAM^+$ periinfundibular region of the ORS (piORS). Analogous to the LFA-1 and ICAM-1 mRNA steady state levels of full-thickness skin homogenates, the number of $LFA\text{-}1^+$ cells was decreased markedly after anagen induction and it increased again during middle and late anagen similar to the ICAM-1 IR of the piORS and the interfollicular dermis. Furthermore, ICAM-1 upregulation on the proximal CTS appeared to be correlated inversely with NCAM expression. $MAC\text{-}1^+$ cells were found throughout the dermis and subcutis which were stained weaker during catagen compared to anagen. The spatiotemporal distribution of ICAM-1 and its ligands suggests a functional role mainly during hair follicle regression. NCAM may join dermal papilla fibroblasts into a functional unit with anagen-inducing properties. NCAM downregulation may play a role in anagen termination. $Pcad^+$ germ cells may represent a topobiologically defined subpopulation of follicular KCs with special sensitivity to inductive signals from the DP.

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SEQUENTIAL ASSEMBLY OF THE HAIR FOLLICLE IMMUNE SYSTEM (HIS) DURING NEONATAL HAIR FOLLICLE DEVELOPMENT IN MICE. R. Paus, U. Hofmann, W. Sterry; Dpt. of Dermatol., Virchow-Hospital, Humboldt-Universität zu Berlin, D-13353 Berlin, Germany

It is unknown in what sequence and under the expression of which surface markers the hair follicle is populated by intraepithelial T cells (DETC; pan-γδTCR+) and Langerhans cells (LHC; NLDC145+ or MHC class II+) during its development. Here, we have characterized the immunohistological appearance of DETC, LHC and intraepithelial CD4+ or CD8+ T cells during neonatal hair follicle development in C57BL/6 mice. The first immunocytes detectable in epidermis on day 1 after birth are predominantly DETC and a minority of MHC II+ LHC. However, these do not migrate into the follicle epithelium until follicle development is almost completed. NLDC145+ LHC, which are not seen in the epidermis during the first 3 postnatal days, also do not enter the follicle epithelium until late in follicle morphogenesis, resulting in a significant increase of intrafollicular NLDC145-immunreactivity (-IR) about day 8. Interestingly the intraepithelial MHC II-IR lags behind. Hardly any intraepithelial CD8+ Tcells are detected during neonatal hair follicle development. The very few intraepithelial CD4+ T cells increase in numbers on day 3 in the epidermis,whereas those located intrafollicularly do apparently not show numeric fluctuations during the first 2 wks. of neonatal life. In contrast, DETC numbers increase highly significantly within the epidermal and distal follicular epithelium during days 5-10. We conclude that by the the time the first hair shafts penetrate the skin, opening a major port of entry for infectious organisms, the relevant cellular constituents of the murine HIS (DETC,LHC) have reached their definite positions.

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ANAGEN-ASSOCIATED SYSTEMIC DEPRESSION OF CONTACT HYPERSENSITIVITY (CHS) IN MICE - A RESULT OF SYSTEMIC IMMUNOSUPPRESSION? U Hofmann, Y Tokura*, M Takigawa*, W Sterry, R Paus; Dpts. of Dermatology, Virchow-Hospital, Humboldt-Universität zu Berlin, D-13353 Berlin, Germany and *Hamamatsu Univ., Hamamatsu, Japan

Depression of CHS reactivity to picrylchloride (PCl) is observed in mice when this sensitizer is applied while major parts of the mouse integument are in the phase of active hair growth (anagen) (JID 106:598,1996). We now have used the C57BL/6 mouse model for hair research to examine whether local immunomodulatory changes in skin cytokine network or systemic immunosuppressive factors which might arise during anagen are responsible for this anagen-associated suppression of CHS. We found that prior depilation of telogen *back* skin, thereby inducing a large skin area with anagen hair growth, 3 days before sensitization to PCl (1%, 50µl) via *abdominal* skin caused significantly ($p < 0.05$) reduced ear swelling-responses to PCl challenge 5 days later. Asking, next, for the influence of hair cycling on the challenge phase, mice were challenged 3 days after anagen was induced by depilation in telogen back skin on day 4 after sensitization. This resulted in a significantly decreased ear swelling response, compared to the non-depilated control group. Using semi-quantitative RT-PCR we could show, furthermore, that IL-10, TNF-alpha and IL-1-alpha mRNA steady state levels in epidermal sheet preparations are increased during anagen. Also, the ratio of epidermal Langerhans cells (NLDC-145+) to DETC (γδTCR+) declines during anagen, which has been suggested to be correlated with weaker CHS responses (JID 89:495, 1987). We speculate that anagen skin generates systemically immunosuppressive cytokines that may down regulate both the afferent and the efferent phase of CHS.

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THE ROLE OF NEUROPEPTIDES IN MURINE HAIR CYCLE MODULATION: INDUCTION OF HAIR FOLLICLE REGRESSION (CATAGEN) BY CAPSAICIN AND SUBSTANCE P. M. Maurer, E. M. J. Peters, E. Fischer, V. Botchkarev, S. Eichmüller, and R. Paus, Dept. of Dermatology, Virchow Hospital, Humboldt-Universität zu Berlin, Germany.

Increasing evidence suggests a role for neuropeptides (NP) in hair growth control, either by direct effects of NP released from skin nerves on follicle cells, or via the activation of mast cells (MC)(Dev Biol 1994; 163:230, Arch Dermatol Res 1994; 287:500). Using the C57BL/6 mouse model for hair research, we have therefore investigated whether capsaicin (CPS) - which depletes NP from sensory nerves, but may also have direct MC-secretagogue properties - or substance P (SP) can induce catagen. Mice with all back skin follicles in the depilation-induced growth phase of the hair cycle (anagen) received injections of CPS (35 mg/kg body weight, on day 12 of the hair cycle), SP (10^{-5}M, on days 10 and 11 of the hair cycle) or the appropriate vehicle into the lower region of the back skin. Catagen development was assessed by documentation of the characteristic catagen-associated skin color change from black to gray-pink and by histomorphometric analysis. Injections of CPS or SP resulted in the induction of localized catagen development in 20 out of 20 and 4 out of 6 mice, respectively, while none of the control animals showed signs of hair follicle regression. When follicles of the treated skin areas were scored by histomorphometry for progression in the hair cycle, a premature entry into catagen of both CPS- and SP-treated mice, as compared to vehicle treated control animals, was found ($p<0.05$). However, most test follicles showed mild signs of dystrophy. Surprisingly, CPS- or SP-treated follicles subsequently ran through a substantially shortened resting phase (telogen), and reentered into the next anagen phase significantly earlier than control follicles. This suggests that exogenously administered NP (like SP) or experimentally released NP (by CPS) can exert profound regulatory effects on hair follicle cycling in mice, either directly or via stimulation of hair growth-regulatory MC-activities.

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EPIDERMAL FATTY ACID-BINDING PROTEIN (E-FABP) EXPRESSION IN THE HUMAN HAIR FOLLICLE. I. Masouyé, J.H. Saurat , G. Siegenthaler, Department of Dermatology, University Hospital, Geneva, Switzerland.

E-FABP is a 15 kDa cytoplasmic protein which is expressed in the epidermis and oral mucosa. E-FABP specifically binds fatty acids (FA) with high affinity and its level is correlated with the intracellular trafficking of FA and keratinocyte differentiation. In normal epidermis, E-FABP expression is restricted to the granular- and uppermost spinous layers. Since data concerning FA metabolism in the hair follicle is scarce, a detailed analysis of E-FABP expression in the adult human hair follicle was performed by immunohistochemistry.

The outer root sheath (ORS) was not stained in the lower part of the bulb; when ORS stratified however, E-FABP expression appeared in its innermost layers. All its suprabasal layers were positive at the stem, isthmus and infundibulum but the bulge was negative. The inner root sheath (IRS) strongly expressed E-FABP before cornification. The hair cortex was stained, although less intensively than the IRS. E-FABP was not detectable in sebocytes.

E-FABP is more widely distributed in keratinocytes of human hair follicle than in the normal epidermis. Its presence in the 3 main compartments of the hair follicle (cortex,IRS,ORS) indicates that there is an intense metabolism of FA in this epidermal appendage. The absence of E-FABP in sebocytes suggests that it may not be important for sebogenesis. The role of FA transported by E-FABP during hair formation remains to be determined.

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INTERLEUKIN-1ß INCREASES 5α-REDUCTASE ACTIVITY IN ISOLATED HUMAN HAIR FOLLICLES. W. Eicheler, R. Happle, R. Hoffmann. Philipp University, Department of Dermatology, Marburg, Germany

Androgenetic hair loss is frequently associated with histopathological signs of perifollicular inflammation. This observation gives rise to the question whether mediators of the inflammation might enhance androgenetic hair loss. The action of androgens in many target tissues depends on the presence of 5α-reductase, a key enzyme of intracellular androgen metabolism. Remarkably, individuals lacking 5α-reductase do not develop androgenetic hair loss. Hence, inhibition of 5α-reductase should be a reasonable approach to treat androgenetic hair loss. In balding men, increased levels of interleukin-1 (IL-1), a potent inhibitor of hair growth both in vitro and in vivo, have been found in hair follicles from the vertex as compared to hair follicles from the nape.

Following this line of thought, we examined the effect of IL-1ß on 5α-reductase activity in isolated human hair follicles. Using a radiochemical assay we found an increased 5α-reductase acitivity after incubation with IL-1ß in single hair follicles obtained from men and women. This increase was antagonized by a molar excess of IL-1 receptor antagonist (IL-1ra). In female hair follicles, that usually express less 5α-reductase than male follicles, 5α-reductase activities were elevated to the male level after IL-1ß treatment. Because an increased 5α-reductase activity results in a higher androgen sensitivity of hair follicles, our data suggest that both inflammatory and endocrine components may act synergistically giving rise to folliclular injury in the form of androgenetic hair loss. The findings could be important regarding the development of new therapeutic approaches for this common condition.

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cAMP IS THE INTRACELLULAR SECOND MESSENGER OF IL-1ß-INDUCED HAIR GROWTH INHIBITION. R. Hoffmann, W. Eicheler, R. Happle, Dept. of Dermatology, Philipp University, Marburg, Germany.

IL-1 has been shown to be a potent inhibitor of hair follicle growth in vitro, and recently we have hypothesized that this cytokine might be a decisive factor causing hair loss during the lymphocytic attack in alopecia areata. Neither the intracellular pathways involved in hair growth inhibition mediated by IL-1ß nor the signal transduction processes within hair follicles in general are known. We therefore investigated the intracellular signals involved in inhibition of hair growth by IL-1ß and in prolonged or augmented hair growth in vitro. Human hair follicles were isolated from scalp biopsies by microdissection, and daily hair growth was measured by image analysis. We assessed intracellular signals transducing elements using specific inhibitors or activators which were used alone or coincubated with IL-1ß.

The calcium ionophore A 23187 induced rapid and complete hair growth arrest, and incubation with phorbol-12-myristate-13-acetate (PMA), genistein or IL-1ß decreased hair growth by approximately 60%-80%. IL-1ß inhibited hair growth, however, was not antagonized by calphostin C, a specific inhibitor of protein kinase C. In contrast, coincubation of IL-1ß with pertussis toxin or H 1004 neutralized the effect of IL-1ß and dbcAMP or cholera toxin, an activator of adenylate cyclase, also inhibited hair growth. These data suggest that cAMP is the second messenger for IL-1ß-induced hair growth inhibition. Moreover, our dat indicate that hair growth in vitro is dependent on intracellular Ca^{2+} levels and activation of tyrosine kinase and PKC.

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cDNA CLONING, SEQUENCING AND ANALYSIS OF EXPRESSION OF 3 TYPE II HUMAN HAIR KERATINS. Rogers M.A.*, Langbein L.§, Prätzel S.§, Krieg T.#, Winter H.*, Schweizer J.*; Research Program 1§ and 2*-German Cancer Research Center, Heidelberg, Germany, Dept. of Dermatology, University of Cologne, Germany#.

As part of a program designed to characterize human hair keratin genes, we present cDNA sequences for three human type II hair keratins, hHb2, hHb3 and hHb5, which, by virtue of their amino-acid homologies, are the orthologs of the previously described type II sheep wool keratins KII-10, KII-11 and KII-12. Amino-acid comparisons of all type II human hair keratins, including the previously published hHb1, show extreme sequence conservation in the amino-terminal, alpha-helical and proximal carboxy-terminal domains. RNA in situ-hybridization of human hair follicles with specific 3' noncoding region probes reveals sequential patterns of expression. The expression of hHb5 mRNA occurs initially in the area of the supramatrix, leaving the cell layer adjacent to the dermal papilla unlabeled, and extends upward into the cortex of the hair shaft. hHb1 and hHb2 cortical expression begins 10-15 layers above the apex of the dermal papilla, thus partially overlapping the expression of hHb5, but continuing into the zone of keratinization to a point well beyond hHb5. hHb3 mRNA expression starts slightly higher than either hHb1 or -2 and proceeds much farther up the hair shaft than either of these keratins. This study demonstrates a three tiered expression of type II hair keratins in the human hair follicle.

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THE INFLUENCE OF FEMALE SEXUAL HORMONES ON SKIN THICKNESS EVALUATED BY 20 MHZ SONOGRAPHY.

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It is well know, that sex hormones influence the skin. Our aim was to investigate changes of skin thickness and density during the spontaneous menstrual cycle, under hormonal contracteptives and during pregnancy.

We measured the skin of 4 groups of women: Group 1 - women with spontaneous ovulatory menstrual cycle, Group 2 - women taking 1-phase contraceptives, Group 3 - women taking 3-phase contraceptives and Group 4 - pregnant women. Skin thickness and echogeneity were measured using the 20 MHz ultrasound system Dermascan C, Cortex Technology, at the following localisations: proximal and distal forearm and lower leg on both sides. The skin was investigated during three phases of the menstrual cycle: phase A (day 2-4), phase B (day 12-14) and phase C (day 20-22). Estradiol and progesterone levels were determined at each phase. The pregnant women were measured 2 weeks prenatal and 6 weeks after birth.

Group 1 showed a statistically significant increase of skin thickness from phase A to phase B, but not from B to C. Group 2 showed now significant changes in skin thickness, whereas in Group C the skin thickness increased from phase A to B. In Group 4 the skin was significantly thicker prenatal than after birth. The measured echogeneity did not correlate to the changes in skin thickness.

In summary we could demonstrate that the status of the female sexual hormones influences the thickness of the skin. These results can be explained by hormone induced water retention in the skin. The 20 Mhz sonography is able to quantify these effects.

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SYSTEMIC THERAPY WITH ESTROGEN OR ESTROGEN WITH PROGESTIN HAS NO EFFECT ON SKIN COLLAGEN IN POSTMENOPAUSAL WOMEN.

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Menopause involves an absolute decrease in the production of estrogen and progesterone. According to the previous studies, the collagen content of the skin decreases after menopause and this decrease is reversed by estrogen.

To investigate the effect of estrogen alone or combined with progestin on the amount and synthesis of skin collagen in postmenopausal women, forty-three early postmenopausal women were enrolled into this open, non-randomized parallel-groups study. Fifteen women received a continuous oral dose of 2 mg of 17β-estradiol and 1 mg of norethisterone acetate daily and 14 women an oral dose of 2 mg estradiol valerate daily. Fourteen subjects served as controls. The histology and type I and III procollagen immunohistochemistry of the skin, skin thickness, the amount of total collagen determined by a colorimetric method and the synthesis of type I and III collagens determined by analysing procollagen propeptides in the suction blister fluid were studied before the treatment and at 6 and 12 months. The proportional area of elastic fibers and the thickness of the epidermis were assessed from the sections obtained before the treatment and at 12 months with computerized image analysis.

Any of these parameters did not change markedly during the follow-up.

In conclusion, one-year treatment with systemic estrogen alone or combined with progestin does not change the amount of collagen or the rate of collagen synthesis in postmenopausal women.

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ASSOCIATION OF LIGHT SENSITIVITY WITH THE ALLELE TNF2 OF THE TUMOR NECROSIS FACTOR-α PROMOTER/ENHANCER IN PATIENTS WITH SCLE

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The genes of the pluripotent cytokines tumor necrosis factor-α (TNF-α) and lymphotoxin-α (LT-α; also called TNF-ß) have been characterized and genetically linked within the HLA class III region of the human major histocompatibility complex. Recently, immunogenetic associations with certain HLA-haplotypes, e.g. A1, B8, DR3 have been described in patients with systemic lupus erythematosus (SLE) and cutaneous LE (CLE). Interestingly, point mutations of the TNF-α promoter (-308 bp, TNF2) and LT-α gene (TNFB*1) were shown to be in linkage disequilibrium with HLA-B8 and -DR3. Furthermore, evidence for genetic association of high response TNF-α secretion *in vivo* with the rare allele TNF2 was found. Genomic DNA of 191 LE patients was isolated and the TNFA and LTA loci were typed with PCR-based DNA techniques. TNF2 allele frequencies of 66 SLE (0.28), 121 CLE (0.28), and 41 patients of the light sensitive subform subacute cutaneous LE (0.35) were significantly elevated compared to the 117 controls (0.16). Moreover, after clinical and histological evaluation standardized photoprovocation testing was applied. The TNF2 allele frequency of photoprovocable SCLE patients (0.41) was significantly associated with light sensitivity ($p<0.0001$). Our observation of genetic differences in the TNF/LT genes associated with a putative high response to UV radiation shows for the first time a genetic link to this hallmark in the pathophysiology of LE.

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ENDOGENOUS RETROVIRAL SEQUENCES IN INTRON 5 OF THE HLA-DRß CHAIN IN PATIENTS WITH CUTANEOUS AND SYSTEMIC LUPUS ERYTHEMATOSUS

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Endogenous retroviral sequences (ERV) may be involved in the pathogenesis of the autoimmune disease lupus erythematosus (LE). ERV are integral parts of the eucaryotic genome showing high homology to the gene structure of exogenous retroviruses. ERV contain long terminal repeats (LTR) bearing elements necessary for gene regulation. One genetic hot spot associated with autoimmune disease is the major histocompatibility complex. Depending on the HLA-DR haplotype ERV9 LTR were found in Intron 5 of the DRB1 and DRB3 genes (Svensson et al., Immunogenetics 1996, in press). Using polymerase chain reaction (PCR) we amplified the genomic region spanning from the 3' end of Exon 4 to the 5' end of Exon 6 in order to investigate ERV9 inserts in patients with systemic and cutaneous forms of LE. We found seven different patterns (a-g), containing a common fragment of 0.8 kb without the ERV9 insertion and identified in addition bands varying in size (1.15 to 1.6 kb) corresponding partly to known ERV9 inserts. Preliminary data indicate that patients with subacute cutaneous LE (SCLE; n=14) have a significant reduction of pattern e (one band, 0.8 kb; 14% vs. 44%) and elevation of pattern c (two bands, 0.8 kb and 1.45 kb; 28% vs. 16%). The genomic sequences are currently determined. In conclusion, ERV inserts in Intron 5 of the HLA-DRß chain in SCLE patients may indicate a new polymorphism possibly involved in HLA gene regulation.

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MEMBRANOUS FAS AND SOLUBLE FAS ANTIGEN IN PATIENTS WITH CUTANEOUS LUPUS ERYTHEMATOSUS

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Since Fas defect was reported to be responsible for the autoimmunity of SLE-prone MRL/lpr mice, Fas/FasL system has been investigated in autoimmune diseases in relation to impaired apoptosis. The role of Fas-mediated apoptosis is still obscure in cutaneous LE. We determined apoptotic cells in skin specimens of SLE and DLE, and in UVB light-irradiated cultured keratinocytes from these patients by using nick labeling method. These studies revealed no differences in apoptotic cell ratio among SLE, DLE and normal controls although cytotoxicity induced by UVBL was higher in cultured keratinocytes from SLE patients than in cells from others. Flow cytometric pattern of Fas on cultured cells were similar among these patients and controls.The next experiment was to evaluate the membranous Fas antigen (mFas) expression on peripheral blood mononuclear cells by flow cytometry, and soluble form of Fas antigen (sFas) in sera. Although the ratio of mFas was not changed among SLE, DLE, eczema and controls, the mean fluorescence intensity in SLE was much higher than others. sFas of SLE was elevated, and the active stage showed the lower value than the inactive stage. The results suggest that the apoptosis is involved in the impaired T cell function,but not in the skin tissue damage.

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AUTOANTIBODIES TO C-REACTIVE PROTEIN AND OTHER ACUTE PHASE PROTEINS IN AUTOIMMUNE DISEASES.

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Autoantibodies to acute phase proteins and in particular to C-reactive protein (CRP) have been reported previously in exogenously induced autoimmune-like disease. We therefore tested sera from patients with systemic lupus erythematosus (SLE n=45), systemic sclerosis (SSc n=42), primary biliary cirrhosis (PBC n=10) and 40 control sera for the presence of antibodies to CRP, α1-antitrypsin (AT), ceruloplasmin and fibrinogen using ELISA techniques. Patients with SLE had in 78% anti-CRP, while patients with SSc had mainly anti-ceruloplasmin (40%). Patients with PBC had the highest and broadest amount of antibodies (anti-CRP 70%, anti-AT 30%, anti-ceruloplasmin 30%, anti-fibrinogen 40%). It is known, that binding of CRP to polystyrene in ELISA plates causes conformational changes exposing non-native regions of the molecule. By comparing serum antibody binding to native versus urea/EDTA denatured CRP preferential binding to denatured epitopes of the CRP molecule was demonstrated in SLE and PBC sera, which could be inhibited in a dose-dependent way. We also compared serological and clinical abnormalities in SLE and SSc patients. A significant elevation of liver enzymes, liver disease and rheumatoid factor could be detected in those patients with SLE and anti-CRP antibodies. Patients with systemic scleroderma and antibodies to ceruloplasmin had no such correlation.We demonstrate for the first time antibodies to cryptic epitopes of CRP and other acute phase proteins in systemic autoimmune diseases. In SLE a correlation between liver disease and CRP antibodies points to the hypothesis, that CRP was either directly denatured or was *de novo* synthesized with conformational changes probably due to inflammatory processes. The clinical relevance of this observation remains to be explored.

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AUTOEPITOPE OF HUMAN CARBONIC ANHYDRASE II MIMICS THE CAPSID PROTEIN OF SPIROPLASMA VIRUS. Masashi Ono, Mariko Ono, Keisuke Watanabe and Hiroaki Ueki. Department of Dermatology, Kawasaki Medical School, Kurashiki, Japan.

Carbonic Anhydrase (CA) is a basic zinc metalloenzyme and it works for regulation of acid–base status primarily in the exocrine organs and erythrocytes. We previously found the autoantibody to this enzyme in sera from patients with systemic lupus erythematodes (SLE) and sjogren's syndrome (SjS). Recent studies have shown that PL/J mice immunized with CA II developed autoimmune sialoadenitis, which indicated that anti–CA II autoantibodies could have a pathogenic role. In this study, to understand detail of this antibody, we have identified linear autoepitopes of CA II. A series of synthetic peptides were originated from primary structure of CA II. Those were coupled with the bovine serum albumin, then coated on 96 well plates. Reactivities of patients' sera to those peptides were analyzed by enzyme–linked immuno sorbent assays (ELISA). As a result, peptide 2, 8 and 15 were recognized by most of anti–CA II positive sera. We also observed an epitope spreading on CA II in sera from a patient with SjS who suffered from renal tubular acidosis. Interestingly, the amino acid sequence of the peptide 15 which is a most prominent epitope have a homology to a capsid protein of the spiroplasma virus, recently it was found to be able to produce persistent infection of a mammal.

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DIFFERENCES IN EXTRACELLULAR MATRIX PROTEINS, EPIDERMAL GROWTH AND DIFFERENTIATION IN DISCOID LUPUS ERYTHEMATOSUS, LICHEN PLANUS, AND THE OVERLAP SYNDROME. E.M.G.J. de Jong, C.J.M. van der Vleuten, I.M.J.J. van Vlijmen-Willems, Department of Dermatology, University Hospital Nijmegen, The Netherlands.

Lichen Planus (LP) and Discoid Lupus Erythematosus (DLE) are separate disease entities. Nevertheless, patients with a so-called 'overlap syndrome' have been described occasionally. The aim of the present study was to establish whether the LE/LP overlap syndrome, based on clinical and routine histological features, could be delineated from DLE or LP using immunohistochemical techniques.

Formalin-fixated, paraffin-embedded skin biopsies of patients with DLE (8), LP (8) and the overlap syndrome (16) were compared regarding immunohistochemical markers for epidermal growth and differentiation and extracellular matrix components. Monoclonal antibodies were used staining cycling cells (Mib-1), keratin 10, keratin 13/16, involucrin, together with antibodies staining extracellular matrix proteins (laminin, heparansulphate and tenascin).

In all diseases, for all markers, changes were observed, compared to normal skin. In DLE and in the LE/LP overlap syndrome the numbers of cycling cells were comparable, but in LP the numbers were significantly lower. Keratins 13/16, 10 and involucrin showed no statistically significant differences between the three disease categories. Heparansulphate and laminin staining was decreased in LP compared to DLE and the LE/LP overlap syndrome. Delineation from the LE/LP overlap syndrome was possible. The difference between LP and DLE was also statistically significant. Tenascin staining was most notably increased in LP. The difference in staining pattern between LP and the LE/LP overlap syndrome was statistically significant.

Only with the markers for extracellular matrix proteins, it was possible to delineate the overlap syndrome from LP. This was not possible for the overlap syndrome and DLE. These findings might indicate that the LE/LP overlap syndrome could be considered as LP-like DLE rather than as a distinct disease entity.

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PLASMA LEVELS OF ENDOTHELIN-1 IN THE PATIENS WITH SYSTEMIC SCLERODERMA(PSS)---CIRCADIAN VARIATION AND EFFECTS OF ELECTRICAL ACUPUNCTURAL STIMULATION--

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Forty-two patients with PSS(Barnett I;14, Barnett II;16, Barnett III;12), 10 with SLE and dermatomyositis(DM), SSD(Scleroderma Spectrum Disorders;12 cases) proposed by Maricq et al and 10 healthy controls(HC) were subjected to examination of plasma levels of endothelin-1(ET-1). The sex ratios (male/female) in the patients with PSS, SLE/DM and HC were 7:35, 4:6 and 0:10, and the range of their ages were 22-74, 19-78 and 33-62 years old, respectively. The plasma levels of ET-1 of SSD, PSS(Barnett I), PSS(Barnett II), PSS(Barnett III), SLE/DM and HC were 1.67±0.37, 2.04±0.58, 2.04±0.68, 1.85±0.41, 1.91±0.7 and 1.31±0.34pg/ml, respectively. There was a statistically significant difference in the plasma levels of ET-1 between each collagen disease(SSD, PSS and SLE/DM) and HC in Student's t test($P<0.05$). Although a statistically significant difference was obtained in the plasma levels of ET-1 between PSS group including one case of DM(totally 7 cases) and HC(6 cases) measured at 6:00, 12:00, 18:00 and 24:00, there was no significant circadian variation of plasma levels of ET-1 as examined at 6:00, 12:00, 18:00 and 24:00 in both of PSS group and HC. After more than three years, with the low frequency electrical acupunctural stimulation for unilateral side of hand/arm(30min.), the plasma levels of ET-1 decreased in all 11 cases of PSS treated. These results may suggest that plasma levels of ET-1 are closely related to pathogenesis of collagen diseases, and the long term treatment with electrical acupuntural stimulation improved the plasma levels of ET-1.

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HIGH-DOSE UVA1 THERAPY IN THE TREATMENT OF PATIENTS WITH LOCALIZED SCLERODERMA. H. Stege, S. Humke, M. Berneburg, K. Dierks, S. Müller-Forte, M. Klammer, M. Grewe, G. Goerz, T. Ruzicka, and J. Krutmann, Department of Dermatology, Heinrich-Heine-University, Düsseldorf, Germany.

For localized scleroderma (LS) various treatments including penicilline and glucocorticosteroids have been employed and found to be unrewarding. A prominent feature of LS lesions is superficial and deep sclerosis of the skin due to increased collagen synthesis. Exposure of normal human skin to ultraviolet A1 radiation (UVA1R; 340-400 nm) was shown to induce collagenase activity in dermal fibroblasts, indicating that UVA1 therapy may be of benefit for LS patients. In this pilot study, 10 patients with histologically proven LS were exposed to single daily doses of 130 J/cm^2 UVA1. After a total of 30 exposures, in all patients sclerotic lesions had softened and decreased in diameter and several plaques had even completely cleared. High-dose-UVA1 therapy led to a significant decrease in thickness (20 MHZ ultrasound, A-mode) and a reduction in echo-rich structures (B-mode) of irradiated plaques, whereas elasticity of skin lesions increased. In unirradiated control plaques, neither clinical nor ultrasound nor elastometric assessment revealed any improvement, indicating that the observed effects were not due to spontaneous involution. Clinical improvement of skin lesions was associated with a 20-fold upregulation of collagenase mRNA levels in UVA1-irradiated plaques (differential RT-PCR). Follow-up studies in 5 patients demonstrated that the beneficial effects were still present 3 months after cessation of high-dose UVA1 therapy in 4 patients, whereas in 1 patient, a partial relapse of skin symptoms was observed. These studies indicate that high-dose UVA1 therapy may be effectively used for LS.

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CLEARANCE OF LOCALISED SCLERODERMA BY LOW-DOSE UVA$_1$ PHOTOTHERAPY.

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Recently, PUVA bath photochemotherapy has been shown to be effective for treatment of localised scleroderma (LS). We now considered whether photosensitation using 8-MOP prior to UVA exposure is mandatory for clearance of skin lesions. Because UVA$_1$ (340-400nm) irradiation has been shown to induce collagenase activity, which can be reduced in fibroblasts of scleroderma lesions, we hypothesised that UVA$_1$ alone can be of benefit for clearance of sclerotic skin lesions. We here report on our first 20 consecutive patients with severe LS, including a 9-year old girl with linear LS resulting in joint contractures, who have been treated with UVA$_1$ phototherapy. In all patients, therapy with so far available conventional treatment modalities had not resulted in any improvement.

UVA$_1$ phototherapy was performed once daily 4 times per week over a period of 6 weeks resulting in a total of 24 irradiations with doses of 20 J/cm^2 at each treatment session. After 24 treatments, in each patient more than 80% of the lesions were completely cleared and no side effects were observed. Clearance of lesions was documented by clinical scoring as well as assessment of skin thickness and skin density by 20 MHz ultrasound and histopathological analysis of representative skin areas.

These results substantiate our hypothesis that low dose UVA1 phototherapy can be highly effective for clearance of sclerotic plaques and even in patients with advanced LS and lesions rapidly growing irrespective of any conventional therapy.

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VH GENE REPERTOIRE OF B CELLS INFILTRATING SALIVARY GLANDS IN TWO PATIENTS WITH SJÖGREN´S SYNDROME. Sylke Gellrich, Astrid Borkowski, Sven Golembowski, Petra Siegel, Wolfram Sterry, Erika Gromnica Ihle, Sigbert Jahn
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In Sjögren´s patients, B cells were found to infiltrate exocrine glands due to sialadenitis and keratoconjunctivitis resulting clinically in a Sicca phenomenon. From phenotype-immunochemical analyses it was suspected that B cells entering salivary glands become activated and start clonal expansion. Therefore, they may act as the producers of antinuclear antibodies (ANA) , Ro(SS-A), La (SS-B) antibodies and the rheumatoid factor, found to circulate in Sjögren´s patients. Since autoantibody producing B cells obtained from spleen or blood were previously described to express a restricted number of somatically mutated immunoglobulin VH/VL genes, we were interested in studying the B cell repertoire in salivary glands for such events.

Two patients with clinical signs and serological markers of Sjögren´s syndrome were chosen for further analyses. RT-PCR amplification of VH and VL genes from mRNA derived from lip biopsies had been carried out using specific FR1 (5´) and JH/JL (3´) primers. A total of 30 VH gene transcripts was cloned and sequenced. It was obvious that in one particular tissue specimen VH genes belonging to different VH/VL gene families were expressed suggesting a polyclonal origin of infiltrating B cells. Furthermore, these variable gene segments were found to be both mutated and germline-identical. Therefore, autoantibody-producing B cells (selected for affinity maturation to autoantigens) as well as other unrelated B cell clones may be present.

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MONITORING OF ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) IN PATIENTS WITH IMMUNOLOGICAL DISORDERS

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Antineutrophil cytoplasmic antibodies (ANCA) are a heterogenous group of autoantibodies with a wide and diverse range of clinical associations. ANCA was first recognized in systemic vasculitis, but the spectrum of subspecificities and ANCA-associated diseases has subsequently increased enormously. In vasculitis, the diagnostic utility of proteinase 3 (PR3)-ANCA and myeloperoxidase (MPO)-ANCA for Wegener's granulomatosis and microscopic polyangitis, respectively, is now well established. The aim of this study was to evaluate the diagnostic and clinical implications of ANCA after testing serum samples from 54 patients with different immunological disorders (14 systemic vasculitis, 11 systemic lupus erythematosus, 12 other autoimmune disorders with vasculitis, 12 abortus habitualis and 5 chronic inflammatory bowel disease) for the presence of these antibodies. The ANCA were detected by means of an ELISA technique (Epignost), anti-PR3 and anti-MPO ANCAs were determined parallel in all patients. Among the patients with systemic vasculitis, we found anti-PR3 or anti-MPO ANCA positivity in 3 and 5 patients, respectively. The results did not reveal a close connection between the occurrence of ANCA positivity and the clinical conditions of the patients. In the SLE group, both types of ANCA were detected in lower frequency. However, it was noteworthy that all patients with a have renal involvement were positive for anti-PR3 ANCA. In the other cases ANCA, could be demonstrated rarely. It was concluded that ANCA investigations are of limited diagnostic and clinical importance, but in some cases may predict complications.

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AUTOANTIBODIES DIRECTED AGAINST FcεRIα: IgG SUBTYPE COMPOSITION, PREVALENCE, AND DISEASE SPECIFICITY. Edda Fiebiger, Sibylle Wichlas, Georg Stingl, and Dieter Maurer, Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases (DIAID), University of Vienna Medical School, Vienna, Austria.

Recently, we identified IgG autoantibodies (autoAbs) directed against the α-chain of FcεRI (FcεRIα) in sera from chronic urticaria (CU) patients. The aim of the present study was to investigate the prevalence of these autoAbs in a larger panel of CU sera and in sera from individuals suffering from other (auto-) immune-mediated skin diseases. In keeping with our previous results, IgG anti-FcεRIα autoreactivity was detected in 37% (36/99) of CU patients but in neither atopic dermatitis patients (0/32) nor healthy individuals (0/35). Whereas IgG anti-FcεRIα Abs were not or only rarely detected in psoriasis (0/30, 0%), bullous pemphigoid (BP; 3/22, 13%) or systemic lupus erythematosus (SLE; 3/15, 20%) sera, these autoAbs were present in a considerable proportion of sera from dermatomyositis (DM; 22/45, 49%) and pemphigus vulgaris (PV; 12/28, 43%) patients. The comparative analysis of IgG subtypes responsible for the observed anti-FcεRIα reactivity in these disease states revealed striking differences. Whereas CU sera contained predominantly IgG1 (12/22, 55%) and IgG3 (17/22, 77%) autoAbs, FcεRIα-specific IgG2 (6/22, 27%) and IgG4 (6/22, 27%) were rarely detected. In fact, only 1 out of 22 CU samples (4%) contained IgG4 autoAbs solely. In sharp contrast, IgG2 and/or IgG4 rather than IgG1 and/or IgG3 were the dominant IgG subtypes responsible for FcεRIα-specific autoreactivity in sera from patients with DM, PV or BP. These differences in the IgG subtypes and, thus, in the complement- and/or Fc receptor-binding properties of these autoAbs are appearantly of functional importance. While it is not yet clear whether the various autoAb isotypes can influence FcεRIα-dependent antigen presentation, we made the striking observation that significant basophil histamine release was elicited by autoAb-containing CU sera but by neither anti-FcεRIα-reactive sera from DM, SLE, BP, or PV patients nor anti-FcεRIα-non-reactive CU sera. As a consequence, therapeutic efforts in CU patients should aim to alter the quality of the anti-FcεRIα immune response occuring in this disease.

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IMMUNOHISTOCHEMICAL INVESTIGATION OF IL-8- AND RANTES-PEPTIDE IN AUTOIMMUNE BULLOUS DISEASES. Erika Bornscheuer, Jens-M. Schröder, Enno Christophers and Michael Sticherling, Dept. of Dermatology, University of Kiel. Kiel, Germany.

Autoimmune bullous diseases of the skin are characterized by local deposition of autoreactive antibodies. Furthermore dermal and epidermal infiltration by neutrophils and eosinophils is initiated by as yet unknown mediators. Members of the recently established chemokine family represent potent chemoattractants for individual leukocyte subsets. IL-8 was found to be neutrophil-chemotactic whereas another peptide, RANTES, is able to activate eosinophils. Accordingly expression of both, IL-8 and RANTES, was monitored by immunohistochemistry to investigate the role of both chemokines. Cryostate sections of bullous pemphigoid (n=12), pemphigus vulgaris (n=3) and IgA-linear-dermatosis (n=2) were examined with a panel of in house and commercial monoclonal (moAb) and polyclonal (pcAb) antibodies. With none of the anti-RANTES antibodies (two moAb and two pcAb) specific immunoreactivity could be detected within either epidermis or dermis of any probe. With the anti-IL-8 antibodies (one moAb and one pcAb) different patterns of immunoreactivity were observed in diseased as well as normal skin. All suprabasal keratinocytes in normal skin were marked with the anti-IL-8 moAb and no significant changes were detectable in bullous skin. With anti-IL-8 pcAb only a slight stainig of basal keratinocytes and some dermal mononuclear infiltrating cells was visible in diseased and normal skin. In conclusion, chemokines cannot be localized within affected tissue. This is in contrast to blister fluids from bullous pemphigoid where elevated IL-8 levels could be detected in the past. Thus, the chemokines may immediately be released from diseased tissue and consequently evade immunohistochemical recovery.

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PEMPHIGUS VULGARIS COEXISTING WITH CICATRICIAL PEMPHIGOID: FLUORESCENCE OVERLAY ANTIGEN MAPPING STUDY. **C. Kowalewski, A. Gorkiewicz-Petkow, and S. Jablonska. Department of Dermatology, Warsaw School of Medicine, Poland**

A 52-year-old woman with oral erosions developed two types of blisters: disseminated flaccid bullae and Nikolsky sign on the trunk and extremities and tense bullae, mainly in traumatized areas, healing with milia formation. Direct immunofluorescence showed IgG and IgM intercellular deposits in the epidermis and linear IgM, IgA and complement (C3) deposits at the dermal-epidermal junction. The in vivo bound IgM, IgA and C3 were found mainly at the dermal side of the patient's split skin, however the weak reaction in epidermis was also observed. Serum studies showed intercellular IgG antibodies, whereas circulating anti-BMZ antibodies were not detectable. Fluorescence Overlay Antigen Mapping was performed on patient's intact skin with the use of monoclonal antibodies against several baseme nt membrane zone antigens: laminin, collagen IV and collagen VII. The overlay image showed yellow linear reaction along the basement membrane due to a complete overlap of green immunofluorescence of in vivo bound C3 deposits and red fluorescence of laminin and collagen IV. The other overlay showed distinctive red collagen VII beneath yellow-orange band of C3. The immunoelectron microscopic studies confirmed the localization of immunodeposits both in lamina lucida and lamina densa suggestive of cicatricial pemphigoid coexisting with pemphigus vulgaris.

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EXPRESSION OF PLASMINOGEN ACTIVATOR RECEPTOR IS ENHANCED BY PEMPHIGUS IgG IN CULTURED KERATINOCYTES. Shiho Satoh, Mariko Seishima, Mari Nojiri, Kazuko Osada, and Yasuo Kitajima, Department of Dermatology, Gifu Univ. School of Medicine, Gifu, Japan.

We have previously reported that pemphigus IgG(P-IgG) causes a distinct increase in plasminogen activator(PA) activity in culture medium for DJM-1 cells. Since secreted PA binds to its receptor on the cell membrane, it is likely that the expression of receptor(PAR) on the cell surface is enhanced by P-IgG, so that PA bound to the cell surface can produce plasmin only in the limited microenvironment around the cells. This plasmin activity generated in the microvicinity around cell surface may cause acantholysis in the limited upper or suprabasal layers characteristic to pemphigus foliaceus(PF) and pemphigus vulgaris(PV), respectively. In order to prove this hypothesis, the effects of IgGs from bullous pemphigoid (BP), PV, PF and normal sera on PAR were examined by Western blot and immunofluorescence microscopy in DJM-1 cells. PV-and PF-IgGs extremely increased the expression of PAR, while BP-and normal IgGs did not. These results suggest that the enhanced PAR expression and PA activity may be involved in the pathogenesis of limited acantholysis specific to PV and PF.

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ULTRASTRUCTURAL LOCALIZATION OF CELL JUNCTIONAL COMPONENTS IN HAILEY-HAILEY DISEASE, DARIER'S DISEASE, AND PEMPHIGUS VULGARIS J.TADA[1], J. ARATA[1] AND K.HASHIMOTO[2]
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The distribution of desmoglein, plakoglobin, and E-cadherin in the peri-lesional and lesional skin of Hailey-Hailey disease, Darier's disease, and pemphigus vulgaris was examined by immunoelectron microscopy using postembedding method.

In the peri-lesional skin the immunolabeling of these desmosomal components was localized to desmosomes. Adherens junction-associated E-cadherin was at the cell periphery excluding desmosomes. The labeling pattern was similar among these diseases, but the labeling intensity particularly that of plakoglobin in Hailey-Hailey disease and Darier's disease, was less than that in pemphigus vulgaris and normal controls. In the acantholytic cells of Hailey-Hailey disease and Darier's disease the immunolabeling of the desmosomal components was diffusely distributed in the cytoplasms. The labeling with an antibody against intracellular component of E-cadherin showed a similar pattern to that of the desmosomal components in these two diseases. The labeling intensity of the desmosomal components was less in Darier's disease than that of Hailey-Hailey disease. In contrast, desmosomes of detaching keratinocytes in pemphigus vulgaris still showed the labeling of desmoglein and plakoglobin.

These findings suggest that inherited acantholytic diseases such as Hailey-Hailey disease and Darier's disease have different pathogenesis from that of autoimmune acantholysis in pemphigsus vulgaris: The intracellular components of both desmosomes and adherens junctions may primarily be disrupted in the genetic acantholytic diseases in the initial stages of acantholysis.

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E-Cadherin Expression is Down-Regulated in a Canine Model for Hailey-Hailey Disease

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Hailey-Hailey (HH) disease is an autosomal dominant genodermatosis characterized by suprabasal acantholysis associated with epidermal hyperplasia. Recently, a canine model for HH disease has been described in an English Setter and his offsprings. To investigate the adhesion defect, we analyzed the expression of several components of the main keratinocyte adhesion structures, adherens junctions and desmosomes, in cell cultures of lesional skin. Northern blot analysis revealed that the steady-state mRNA level of E-cadherin but not of α-catenin, β-catenin, plakoglobin, nor desmoglein 1 was downregulated in cell cultures of HH lesions when compared to those of normal canine skin. This decreased E-cadherin mRNA expression was further paralleled by a decreased expression of E-cadherin protein, as shown by flow cytometry. Interestingly, the expression of E-cadherin appeared heterogeneous in immuno-histochemical analysis of HH cell cultures: small basaloid cells were devoid of E-cadherin expression, whereas larger suprabasaloid cells expressed comparable levels of protein than normal keratinocytes. To further substantiate this observation we analyzed sections of lesional skin as well as lip epithelium of the English Setter. Consistently with the observations obtained for cell cultures, reduced levels of E-cadherin expression were observed in oral basal cells. Surprisingly, E-cadherin expression was absent in all layers directly adjacent to the lesion. Expression of α-catenin, β-catenin, plakoglobin and desmoglein 1 protein was similar in affected tissue and normal control tissue. In summary, our results indicate that in this model of HH disease cellular adhesion is impaired by the downregulation of E-cadherin expression.

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IDENTIFICATION OF SEQUENCES IN THE CYTOPLASMIC DOMAIN OF HUMAN BULLOUS PEMPHIGOID ANTIGEN 180 (hBP180) REQUIRED FOR ITS RECRUITMENT INTO HEMIDESMOSOMES (HD).

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BP180 is a component of HD, multi-protein complexes that mediate adhesion of epithelial cells to the underlying basement membrane. To define regions involved in HD assembly, we have expressed full-length hBP180 and deletion mutant forms of this protein in the rat bladder epithelial cell line 804G. hBP180 was correctly targeted to HD, codistributing with BP230 and the α6 and β4 integrin subunits, markers of the hemidesmosomal plaque. Chimeric cDNA constructs encoding a membrane targeting signal (K-Ras) and the entire cytoplasmic tail of hBP180 or a cytoplasmic tail with increasing internal truncations were also correctly incorporated into HD. However, a hBP180 chimeric protein lacking a cytoplasmic stretch of 289 amino acids including the four tandemly arranged 24 residue repeats remained diffusely distributed on the cell surface. Finally, a hBP180 construct lacking the whole extracellular domain and the transmembrane region was recruited into HD, although a cytoplasmic accumulation of this molecule was also observed. In COS-7 cells transfected with human α6A and β4A cDNA, the hBP180 chimeric construct encoding the membrane targeting signal (K-Ras) and the entire cytoplasmic domain colocalized with α6β4 integrin and with plectin. In conclusion, in contrast to previous studies, our findings indicate that the cytoplasmic domain of hBP180 harbors functional domains necessary and sufficient for HD targeting. The further characterization of relevant sequences within hBP180 will extend our understanding of mechanisms involved in HD formation.

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DISTINCT BASEMENT MEMBRANE ZONE ANTIGENS ARE RECOGNIZED BY THE CICATRICIAL PEMPHIGOID SERA.

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Cicatricial pemphigoid (CP) is a chronic subepithelial blistering disease of mucous membranes in which lesions often heal with scar formation. The 180kD-bullous pemphigoid antigen (BPAg2) [Bernard et al, 1992], the α3-subunit of laminin-5 (200&165kD) [Kirtschig et al, 1995], and a 168kD mucosal antigen (M168) [Ghohestani et al, 1995] were found to be recognized by the CP sera.

The aim of this study was to further determine the relationship between these basement membrane zone antigens identified by different groups in CP. We applied the immunoblot assay using multiple antigenic sources: human epidermal, dermal, and buccal mucosa extracts and also the purified placental laminin-5, for detection of antibodies specific for the most, if not all, of the epidermal and dermal antigens particularly; BPAg2, laminin-5, and M168. The proteins were separated under reducing conditions in 6% polyacrylamide gel electrophoresis. Among the 19 CP sera, seven recognized the epidermal antigen of 180 kD co-migrated with the BPAg2 identified by a polyclonal anti-BPAg2 antibody. Five additional sera labeled the laminin-5: four reacted with the α3 (200kD) and one with the β3 (140kD) chains that co-migrated with the identical bands labeled by a rabbit polyclonal antibody raised against the laminin-5. Sera from four other patients that did not react with the laminin-5, epidermal or dermal protein extracts, did label a 168kD antigen on the mucosal extracts. Immunoaffinity purified antibodies from the M168 did not further react with the BPAg2 or laminin-5. This study demonstrates that there are different entities presenting as CP; accordingly the diagnosis of CP should take into account the nature of the involved antigen.

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DEVELOPMENT OF AN ELISA SYSTEM FOR THE RAPID DIAGNOSIS OF BULLOUS PEMPHIGOID

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The bullous pemphigoid (BP) autoantigen structures and their amino acid sequences are well known. We have addressed the question of whether the demonstration of circulating antibodies against major (BPAG1, 230 kD) and minor (BPAG2, 180 kD) antigens is possible by means of an ELISA technique with synthetic antigenic peptides. With the help of PeptideStructure software, two-two matched antigenic epitopes were chosen and synthetized. The sera of 33 patients with BP were investigated in parallel by an immunoblot technique using a human epidermal extract, and by an ELISA technique with synthetic peptides. The sera of 16 healthy persons and 10 patients with other bullous diseases served as controls. Among the 33 patients with BP, 24 sera proved positive for at least one synthetic peptide. Positive reactions with the major BP antigen were found in 21 patients by the immunoblot technique and in 20 patients by the ELISA technique with the synthetic peptides, positive reactions were found in 9 patients by the immunoblot technique and 18 patients by the ELISA technique using the synthetic antigenic epitopes of the minor BP antigen. With the exception of one person all control sera were negative for both antigens in ELISA investigations. In 3 patients with pemphigus vulgaris, characteristic bands against pemphigus vulgaris antigen were identified by means of the immunoblot technique, but all other cases were negative. It is suggested that the development of this technique may lead to an opportunity for the rapid and simple diagnosis of BP.

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PEMPHIGOID NODULARIS. S. Kawana[1], T. Hashimoto[2], A. Ito, K. Sugaya, and H. Arai[3] [1]Div. of Dermatology, St. Luke's International Hospital, Tokyo, [2]Dept.of Dermatology, Keio Univ. School of Medicine, Tokyo, and [3]Div. of Dermatology, the City of Yamato Hospital, Kanagawa, Japan.

We report 45- and 61-year-old women with generalized prurigo nodularis-like eruption whose clinical and immunopathologic features were consistent with the diagnosis of pemphigoid nodularis. In one case, nodular lesions preceded the onset of generalized blistering by two years and in the other, no definite blister nor erosion was seen except for some appearing on the soles during the course of the disease. We investigated the antigen profiles by reaction with the patient sera in the course of the disease using immunoblot analysis. Western immunoblotting of EDTA-separated epidermal extract revealed that only the 230-kD bullous pemphigoid (BP) antigen was recognized by circulating autoantibodies. The 180-kD BP antigen was recognized by immunoblotting of the recombinant protein of BP180 NC16a domain which shows high detection sensitivity. When the reactivity of the sera obtained before and after the development of bullous lesions with the NC16a domain was compared, no particular change was noted. These findings suggest that the onset of bullous lesions in pemphigoid nodularis as well as the clinical appearance of hyperkeratotic nodules is determined by factors other than changes in the serum reactivity with the BP antigens detected by our conventional immunoblotting technique.

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DOES INTRAVENOUS FUROSEMIDE CAUSE BULLOUS PEMPHIGOID ?

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Bullous pemphigoid (BP) is an acquired autoimmune subepidermal blistering disease. Certain drugs are risk factors for induction of BP. The association between BP and intravenous furosemide is rarely reported and has been challenged by a recent study (Bastuji-Garin S et al. Drugs associated with BP. Arch Dermatol 1996;132:272-6). We present a patient with BP after the administration of intravenous furosemide and review the literature on this topic.

A 78 year old man with hypertension, type II diabetes mellitus, rheumatoid arthritis, and coronary artery disease awoke with tense, painless, nonpruritic blisters on his arms and legs 24 hours after being treated with intravenous furosemide for congestive heart failure. He was also taking chlorpropamide, metoprolol, nifedipine, acetylsalicyclic acid, oxybutynin and ibuprofen, all of which were immediately discontinued. A punch biopsy of right antecubital fossa skin revealed a subepidermal vesicular dermatitis with a mixed neutrophil and eosinophil infiltrate, which by direct immunofluorescence showed C3 and IgG linear deposits at the dermal epidermal junction.Skin lesions and biopsy were typical of BP, for which our patient was treated with prednisone and methotrexate. His diabetes intermittently worsened and he also became thrombocytopenic due to methotrexate. After 9 weeks, however, his lesions healed without superimposed infections and he was discharged.

A review of the literature revealed only 6 previously reported cases of BP induced by furosemide. All of these cases were elderly patients suffering congestive heart failure. The daily dose of furosemide varied from 40 mg to 120 mg. The bullous skin lesions were treated either symptomatically or with high dose corticosteroids which may be particularly risky for the elderly. They are often bedridden and prone to develop deep venous thrombosis, pulmonary embolism, and/or infections. Of the drugs our patient was taking, the only ones which were listed as inciters of BP besides furosemide were ibuprofen and acetylsalicyclic acid. It is less likely that BP was caused by either ibuprofen, which the patient took only rarely, or acetylsalicyclic acid, which he took for many years. Our case and previous reports suggest a relationship between BP and intravenous furosemide which needs to be confirmed by prospective case-control studies of a reasonable size.

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IMMUNOHISTOCHEMISTRY WITH AN ANTI-TYPE IV COLLAGEN MONOCLONAL ANTIBODY AS AN AID FOR DIFFERENTIATING BETWEEN BULLOUS PEMPHIGOID AND EPIDERMOLYSIS BULLOSA ACQUISITA.

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Each of methods currently used for differentiating between bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA) has its limitations. Therefore, we evaluated whether immunohistochemistry with an anti-type IV collagen monoclonal antibody could be used for this purpose.

Paraffin-embedded blister-containing biopsy specimens obtained from 8 BP, 2 EBA, 2 dermatitis herpetiformis (DH), 2 lichen sclerosus et atrophicus bullosus (LSAB) and 1 localized recessive dystrophic epidermolysis bullosa inversa (LRDEBI) patients were examined by an avidin-biotin-peroxidase complex (ABC) method. Sera were checked by indirect immunofluorescence (IF) on salt-split normal human skin and immunoblotting (IB) of normal human epidermal and dermal extracts, and NC16a domain of the BP180.

All BP cases and DH control cases had type IV collagen staining at the base of a blister. One EBA case had type IV collagen staining at both the roof and base of a blister, whereas the other did not exhibit clear type IV collagen staining. LSAB control cases and the LRDEBI control case had type IV collagen staining at the roof of a blister. The results of the ABC method corresponded to results of IF and IB in all BP and EBA patients who exhibited clear type IV collagen staining.

Thus, the ABC technique with the anti-type IV collagen monoclonal antibody can be used as an aid for distinguishing BP from EBA in individual cases. However, its use in routine diagnostic work is hampered by the fact that an undamaged epidermal basement membrane has to be present in a biopsy specimen.

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IMMUNOELECTRON MICROSCOPIC ANALYSIS OF DOMAIN-SPECIFIC ANTIBODIES SUGGESTS THAT HUMAN SKIN ANCHORING FIBRILS INITIATE AND TERMINATE IN THE LAMINA DENSA

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The anchoring fibrils at the dermo-epidermal junction are mainly composed of type VII collagen (COLVII) and play a crucial role in the dermo-epidermal adhesion. Previous studies have suggested the anchoring fibrils originate in the lamina densa, extend perpendicularly into the dermis and insert into amorphous elements called "anchoring plaques" in the dermal connective tissue. To elucidate the precise structural organization of the anchoring fibril network in human skin, we have quantitatively analyzed the distribution of different domains of COLVII in the epidermal basement membrane zone using various techniques of immunoelectron microscopy with a range of domain specific antibodies we have prepared. Surface immunolabeling on sections from Lowicryl K11M and cryoultramicrotomy demonstrated that 95% of the epitopes in the aminoterminal of COLVII localized to the lamina densa, and the epitopes in the carboxyterminal end of COLVII localized 300 nm -400 nm distant from the lamina densa. 95% of the epitopes in the central triple-helical collagenous domain distributed between the lamina densa and the 400 nm distant from the lamina densa, and no specific labelings were observed beneath this area. So called "anchoring plaques", were recognized only by pre-embedding en bloc labeling, but not by post-embedding section labeling. These results suggest that most anchoring fibrils in human skin do not extend perpendicularly into the dermis, but they initiate and terminate in the lamina densa forming individual semicircular loop that constitute anchoring fibrils network.

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CHARACTERIZATION OF A NOVEL COMPONENT OF EPITHELIAL BASEMENT MEMBRANES USING GDA-J/F3 MONOCLONAL ANTIBODY

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Using the monoclonal antibody GDA-J/F3, a novel component of epithelial basement membranes was identified. Immunofluorescence stainings of normal human skin and some other stratified epithelia with the GDA-J/F3 antibody showed a linear fluorescence decorating the basement membrane zone. With immunoelectron microscopy, the epitope was localized to the insertion points of the anchoring fibrils into the lamina densa. The antigen is distinct from collagen VII, the main structural component of the anchoring fibrils, since the GDA-J/F3 antibody does not react with purified collagen VII *in vitro*. In serum-free cultures, the antigen was expressed by normal human keratinocytes and epithelial cell lines, and to a lesser extent by normal human skin fibroblasts. Immunoprecipitation of radiolabelled epithelial cell-conditioned medium with the GDA-J/F3 antibody yielded two polypeptides that migrated on SDS-PAGE with apparent molecular mass of 46 kDa and 50 kDa under non-reducing conditions. Using reducing gels, only the 50 kDa polypeptide was observed. The antigen was resistant to digestion with bacterial collagenase, but sensitive to trypsin and pepsin. It also exhibited binding to heparin. These findings indicate that the GDA-J/F3 antigen is a small globular protein with a potential to interact with basement membrane proteoglycans. The antigen is likely to be anchoring fibril-associated, since the GDA-J/F3 epitope was missing in several patients with a genetic blistering disorder of the skin, epidermolysis bullosa dystrophica, who lacked anchoring fibrils.

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ISOLATION OF COLLAGEN XVII / BP180 FROM HUMAN KERATINOCYTES AS A NATIVE COLLAGEN.

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Collagen XVII is a hemidesmosomal autoantigen of pemphigoid diseases and the candidate gene in generalized atrophic benign EB (GABEB). It belongs to the group of novel transmembrane collagens in type II orientation. The cDNA sequence shows intracellular globular sequences, and an extracellular, multiply interrupted collagenous domain. With this unusual structure the molecule represents a new class of epithelial adhesion molecules with characteristics of an integrin and of an extracellular matrix ligand. Using non-ionic detergents, we isolated native collagen XVII from the membrane fraction of human keratinocytes and HaCaT-cells. On SDS-PAGE the molecule migrates with an apparent molecular weight of 180 kD. Immunoprecipitation showed no evidence for a second polypeptide chain, suggesting that collagen XVII is a homotrimer. Digestion by bacterial collagenase resulted in degradation of the collagenous domain, leaving an about 70 kD collagenase resistant fragment. Pepsin digestion with enzyme concentrations usually employed for fragmentation of collagens resulted in complete degradation, indicating low stability of the collagen triple helix. However, limited digestion with extremely low amounts of pepsin yielded the intact extracellular collagenous domain of about 100 kD. Analysis on reducing and non-reducing SDS-PAGE showed that the non-collagenous domain contains disulphide bonds, but the collagenous ectodomain not. Digestion with N-glycosidase F removed N-linked carbohydrates and resulted in an apparent mobility shift of about 5 kD on SDS-PAGE. Autoimmune sera of patients with bullous pemphigoid, herpes gestationes or cicatricial pemphigoid reacted strongly with collagen XVII, but not with other epithelial antigens. Epitope mapping showed that the sera recognized antigenic sites both in the non-collagenous and the collagenous sequences. Characterization of native authentic collagen XVII/BP180 is necessary for understanding the normal functions of the molecule and the pathogenetic mechanisms involved in blistering skin diseases.

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A NEW SUBSET OF JUNCTIONAL EPIDERMOLYSIS BULLOSA IS CHARACTERIZED BY THE ABSENCE OF IMMUNOREACTIVITY OF THE ANCHORING FILAMENT PROTEIN LADININ-1

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Generalized atrophic benign epidermolysis bullosa (GABEB) is a mild form of junctional epidermolysis bullosa caused by mutations involvong laminin-5 and collagen XVII (BP 180). Recently a new 120 KD anchoring filament, Ladinin-1 (LAD-1) has been identified using sera obtained from patients suffering from linear IgA dermatitis (LAD). Using a panel of monoclonal antibodies specific to the dermal-epidermal junction, we have studied skin biopsies from a cohort of 20 GABEB patients. A defective expression of laminin β3 chain was detected in five patients, whereas the remaining patients revealed an altered immunoreactivity of BP 180 and LAD-1. Shortly after birth, three of these patients with a negative staining of LAD-1 and BP 180, had a generalized and severe eruption of blisters related to an extreme fragility of the epidermis. These manifestations were clinically indistinguishable from the hallmarks of the Herlitz syndrome (H-JEB), the most severe form of junctional epidermolysis bullosa, linked to absent expression of the anchoring filament component laminin-5. Electron microscopic examination of skin biopsies in these three patients showed presence of rare and highly rudimentary hemidesmosomes, which is also a characteristic of H-JEB. However, in spite of the dramatic onset of the disease, a rapid recovery of the lesions and a normal growth rate of the patients were then observed, which are not consistent with a diagnosis of H-JEB. These results indicate that the anchoring filament component ladinin-1 may constitute the candidate gene in a subset of GABEB patients with a severe onset but with favourable prognosis. We are currently searching for the possible mutations in the gene for LAD-1 in these patients.

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PROTEOLYTIC PROCESSING OF MEMBRANE-TYPE MATRIX METALLO-PROTEINASE-1 (MT-MMP-1).

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We have recently found that the lysates of untreated and PMA or Con A treated human fibroblasts and fibrosarcoma cells contain the membrane-type metalloproteinase-1 (MT-MMP-1) protein, the postulated cell surface activator of 72-kDa gelatinase, of about 63-kDa, 60-kDa and in some cases 43-kDa in size. To understand the interrelationships between MT-MMP-1 and the activation of 72-kDa gelatinase we have carried out analyses of the processing of MT-MMP-1 by immunoblotting, immunoprecipitation and activity measurements using cultured human fibroblasts and fibrosarcoma cells as models. Antibodies were raised against immunogens corresponding to extracellular and intracellular domains of MT-MMP-1. Immunoprecipitation and immunoblotting analyses indicated that MT-MMP-1 is synthesized as a 63-kDa protein which is processed to a 60-kDa form. In fibrosarcoma cells MT-MMP-1 was further processed to a 43-kDa form, when overexpressed or the cells were treated with PMA or ConA. The processing could be prevented by calcium ionophores. By surface labelling studies we found that all three forms of MT-MMP-1 were present at the cell surface. Analysis of MT-MMP-1 immunoprecipitates from Triton-X100 extracts of fibroblasts by SDS-PAGE followed by immunoblotting for 72-kDa gelatinase or zymography indicated that these two metalloproteinases are associated with each other at the cell surface. Zymographic analyses indicated that the fibroblasts express activated, lower mol. wt. 72-kDa gelatinase (69-kDa) at their cell surfaces in complex with MT-MMP-1, whereas medium samples of the respective cells contained unprocessed 72 kDa gelatinase only. The processing was inhibited under conditions where the processing of MT-MMP-1 is prevented. The results indicate that cell surface-associated activation of 72-kDa gelatinase is tightly associated with proteolytic processing of MT-MMP-1.

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POSITIVE REGULATION OF COLLAGENASE (MMP-1) GENE EXPRESSION BY SPHINGOMYELIN PATHWAY IN HUMAN SKIN FIBROBLASTS. N. Reunanen, J. Westermarck, L. Häkkinen*, and V-M. Kähäri. Dept. of Dermatology and Periodontology*, Univ. of Turku, Finland.

Inflammatory cytokines TNF-α and IL-1 induce expression of collagenase (MMP-1) and stromelysin-1 (MMP-3) in dermal fibroblasts. Both TNF-α and IL-1 trigger the sphingomyelin signaling pathway, initiated by neutral sphingomyelinase (SMase)-dependent hydrolysis of cell membrane phospholipid sphingomyelin to ceramide, which then acts as a second messenger. We triggered this pathway in human skin fibroblasts by treatment with SMase or with synthetic ceramides -2, -6, or -8. Treatment of cells with SMase and ceramides enhanced expression of MMP-1 mRNA and production of immunoreactive MMP-1 in a dose-dependent manner. The ceramide-dependent induction of MMP-1 mRNA levels was abrogated by dexamethasone and cycloheximide, and by tyrosin phosphatase inhibitor orthovanadate. Treatment of cells with SMase and C2-ceramide also radiply and transiently induced *c-jun*, *junB* and *c-fos* mRNAs and increased AP-1 binding on gel shift assays. The mRNA levels of type I collagen were not changed by treatment with ceramides or SMase. These results indicate that the sphingomyelin signaling pathway positively regulates MMP-1 but not type I collagen gene expression in dermal fibroblasts. It is conceivable that targeted modulation of sphingomyelin pathway may offer novel strategies for regulation of collagenolytic activity in inflammatory skin disorders.

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SEEDING NORMAL FIBROBLASTS IN 3-DIMENSIONAL COLLAGEN MATRICES INDUCES APOPTOSIS. Juliane Fluck, Christiane Querfeld, Thomas Krieg, and Stephan Sollberg, Dept of Dermatology, University of Köln, Köln, Germany.

Since cell-matrix interactions have major effects on a variety of biological features such as gene regulation, differentiation, and growth control, we studied the influence of extracellular matrix components on the induction of apoptosis in fibroblasts. Seeding normal primary fibroblasts in 3-dimensional collagen gels (free floating and anchored collagen matrices) clearly induces apoptosis. In more detail, quantitation of the cytoplasmatic histone-associated DNA fragments using a cell death detection ELISA revealed an increase of DNA-fragmentation in both systems starting at day one with a peak at day two. For the detection of apoptotic cells in frozen gel sections, the fragmented DNA in the sections was labeled with terminal transferase and ddUTP-FITC. On day 3 of cultivation we found that approx. 20 % of all visible cells are apoptotic. We conclude that the induction of apoptosis in fibroblasts seems to be specific for 3-dimensional collagen matrices, since apoptosis is not induced in primary fibroblasts grown as monolayers on plastic dishes coated with collagen or on uncoated culture dishes. However, it remains to be elucidated, whether 3-dimensional cell-matrix (i.e. collagen) interactions induce apoptosis of fibroblasts through mechanical forces exerted in these systems or whether apoptosis is dependent from the proliferative state of the cells.

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PDGF ISOFORMS CONTROL THE SPATIAL ORGANIZATION OF CONNECTIVE TISSUE Sabine A. Eming, Rick Snow, Martin L. Yarmush, Jeffrey R. Morgan Shriners Burns Institute/Surgical Services, Massachusetts General Hospital/Harvard Medical School, Boston, MA

Platelet-derived growth factor (PDGF) is a potent mitogen and chemoattractant for mesenchymal cells. PDGF occurs as multiple isoforms, which are either secreted or remain cell associated and human keratinocytes of the epidermis synthesize both types of PDGF. To investigate the *in vivo* role of secreted versus cell associated isoforms of PDGF, retroviral mediated gene transfer was used to introduce into human keratinocytes the genes encoding wild type PDGF-B, which is predominantly cell associated, or a truncated mutant (PDGF-B-211), which is predominantly secreted. Cells expressing the mutant isoform secreted 20 times more PDGF into the culture medium (145 ng/hr/10^7 cells) than cells expressing the wild type isoform, PDGF-BB (6ng/hr/10^7 cells). Both had similar amounts of PDGF on the cell surface (wild type 263 ng versus mutant, 222 ng/5x10^6 cells). When cells were grafted as epithelial sheets onto athymic mice, modified cells formed a stratified epithelium and induced a subjacent connective tissue response which differed depending on the PDGF isoform expressed. Expression of the secreted mutant PDGF-B-211 induced a connective tissue which was thicker then controls and had increased numbers of fibroblasts, mononuclear cells and blood vessels, evenly distributed throughout the entire connective tissue layer. In contrast, expression of the cell associated wild type PDGF-B induced increased numbers of fibroblasts and mononuclear cells which were localized to a zone at the interface of epidermis and connective tissue and often disrupted the continuity of the basement membrane. Immunostaining revealed that wild type PDGF was deposited in the basement membrane region. These data suggest that the different secretory properties of PDGF isoforms control the spatial organization of cellular events in regenerating mesenchymal tissue *in vivo*.

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REDUCED TYPE III COLLAGEN METABOLISM IN HUMAN SKIN FIBROBLAST CULTURES IN JUVENILE HYALINE FIBROMATOSIS.

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Juvenile hyaline fibromatosis (JHF) is inherited as an autosomal recessive disorder characterized by multiple tumorous proliferations of fibroblast-like cells. In order to investigate the role of collagen type III in this disease, we performed fibroblast cultures from normal skin of a patient with JHF. We analysed the supernatants of cell cultures at confluence stage for amino terminal propeptide of type III collagen, regarded as parameter for the overall metabolism of type III collagen (TIIIC) with a radio immuno assay. Additionally skin biopsy specimen were obtained for western-blot analysis of TIIIC. Controls were performed with age matched controls.

We found overall TIIIC metabolism significantly reduced for 40% ($p<0,001$) compared to controls. Western blot analysis showed the absence of immunoreactive collagen type III. In contrary the high molecular weight bands representing cross linked collagens present in polymers, did not show any differences in comparison to the control group. These findings indicate 1) a decreased TIIIC metabolism and/or 2) a possible cross linking deficit of TIIIC involved in JHF.

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Determination of the type XIV collagen domains involved in the binding of procollagen I N-proteinase. A. C. Colige, Y. L. Goebels, B. Y. Nusgens and C. M. Lapière – Laboratory of Connective Tissues Biology, University of Liège, Belgium

Procollagen I N-proteinase (PCI-NP), a newly recognized member of the metallo-proteinase family, is responsible for the excision of the amino-propeptide of type I and type II procollagens. A reduction of its activity results in animal dermatosparaxis and in human Ehlers-Danlos type VIIC, heritable disorders characterized by the accumulation of polymers of p-N-I collagen mainly in the skin and by a severe cutaneous fragility. We have shown that PCI-NP is immobilized on the extracellular matrix and can be co-purified with type XIV collagen. This interaction was further investigated in solid-phase assays. The binding between PCI-NP and type XIV collagen was specific as compared to the low binding observed with other macromolecules. It was abolished by traces of heparin, ionic detergents and by high ionic strength but not by acidic (Glu, Asp) or basic (Arg) amino acids, in monomeric or in polymeric forms. Type XIV collagen digested by pepsin or by bacterial collagenase lost its capacity to immobilize the enzyme but the digestion fragments remained strong inhibitors of binding to native type XIV collagen. Heparin-sepharose chromatography of the polypeptides after pepsin or collagenase digestion allowed the characterization of two independent domains acting as competitors in the solid-phase assay. One is a collagen domain as it is pepsin-resistant but collagenase- and heat-sensitive while the second is a non-collagen domain, probably involving the heparin-binding region of the NC1 domain. We propose that, in vivo, two independent domains of type XIV collagen are required to immobilize PCI-NP in a close vicinity of type I collagen fibers, allowing the spatially controlled processing of the procollagen molecules.

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TOPICAL TRETINOIN INCREASES EPIDERMAL THICKNESS AND INDUCES TENASCIN IN SUN PROTECTED SKIN, BUT HAS NO MAJOR EFFECTS ON SKIN COLLAGEN.

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Topical retinoids have been shown to reduce skin wrinkling on actinically damaged skin by inducing new connective tissue. The action of retinoids on skin in sunprotected areas is not entirely known. In the present study the effects of topical tretinoin on the histological and biochemical parameters were studied in eleven male volunteers (mean age 63 years). The subjects applied 0.1 % tretinoin once a day for two months on the abdominal skin, and vehicle on the other side of the abdominal skin. After two months skin thickness was measured with ultrasound, and skin samples were taken for histological and biochemical assays. In addition suction blisters were raised for the collection of blister fluid to determine collagen propeptides as well as gelatinases.

The overall skin thickness was in tretinoin treated skin 2.46 ± 0.31 mm and in vehicle treated skin 2.25 ± 0.23 mm and the epidermal thickness in vehicle treated skin 55 ± 15 μm and in tretinoin treated skin 146 ± 27 μm. The staining with anti-tenascin showed more intensive signal in tretinoin treated skin in compared to vehicle treated skin. In situ hybridization revealed increased levels of tenascin mRNA in tretinoin treated skin. The measurement of specific mRNAs of α1(I) collagen and several matrix metalloproteinases (MMP-1, MMP-2, MM-9) by slot-blot hybridization did not reveal any significant differences between tretinoin and vehicle treated skin.

In conclusion results indicate that topical tretinoin induces thickening of epidermis and accumulation on tenascin in sun-protected areas but has no major effect on skin collagen metabolism.

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CD7-NEGATIVE T CELLS REPRESENT A SEPARATE DIFFERENTIATION PATHWAY IN A SUBSET OF POSTTHYMIC HELPER T CELLS

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Absence of the CD7 protein and the corresponding mRNA is a stable feature in a subset of normal circulating CD4+ memory T cells. It is still unresolved whether the CD7- subset represents a specific T cell lineage. Here we show that repeated stimulation of highly purified CD4+CD45RA+CD45RO- naive T cells in vitro leads to the development of a distinct memory subset that is defined by the expression vs. non-expression of the CD7 antigen. Comparing different T cell activation pathways (TCR/CD3, CD2) we observed that alternative signals are critically involved in the development of CD4+CD7- T cells. Peak mean numbers of CD7- memory cells occured after 3 to 5 cycles of restimulation in vitro. Naive T cells which had undergone repeated stimulations were harvested and sorted into CD7+ and CD7- subsets. The vast majority (> 97 %) of CD7+ T cells retained their expression whereas the CD7- population did not reexpress the antigen during further propagation of separated T cell subsets. In CD7- cells no CD7 mRNA was monitored indicating transcriptional regulation of CD7 expression. Certain differentiation-related antigens including the cutaneous lymphocyte antigen CLA are preferentially expressed on CD7- T cells. We suggest that absence of CD7 expression in a subset of CD4+ memory cells reflects a separate and stable differentiation state occuring late in the immune response. These T cells may represent the physiologic counterpart of malignant T cells in certain forms of cutaneous T cell lymphoma.

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T CELL RESPONSES TO THE EXTRACELLULAR PORTION (EC1-5) OF DESMOGLEIN 3 (DSG 3) ARE RESTRICTED BY POLYMORPHISMS OF THE β1-CHAIN OF HLA-DR AND BY HLA-DQ MOLECULES.

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CD4+ T cell lines and clones from a patient (HLA-DR11,14; DQ5,7) with pemphigus vulgaris (PV), (PV8; n=7) and a healthy control (HLA-DR11; DQ7), (C11; n=4) were stimulated by the recombinant protein PVhis (EC1-5 of Dsg3 linked to histidines) with HLA-DR11+ DQ7+ peripheral blood mononuclear cells (PBMC) as antigen presenting cells (APC), (stimulation index (SI)=2.6-271). To determine whether DR11 on the APC restricted this T cell response to Dsg3, murine L cells transfected with one of four DR11 alleles were used as APC. T cell lines and clones from PV8 were not stimulated by PVhis with DR11+ L cells as APC (SI=0.7-1.8). The PVhis-driven proliferative response of these T cell lines and clones with DR11+ DQ7+ PBMC as APC (SI=5.1-43) was strongly inhibited by mab against HLA-DQ (SI=0.4-0.8), but not by mab against DR or DP (SI=8.5-43.7), which strongly suggested that HLA-DQ was the restricting element. A T cell line from C11 was stimulated by PVhis with all of the DR11+ L cells as APC (SI =2.3-13.5). T cell clone C11.19 derived from this T cell line was stimulated by PVhis only with DRβ1*1101+ and DRβ1*1104+ (which share phenylalanine at pos. 67 and arginine at pos 71) L cells as APC (SI =3.9-21.5). Clone C11.32 was stimulated by PVhis with DRβ1*1102+ and DRβ1*1103+ (sharing glutamic acid at pos. 71 and valine at pos. 86) L cells as APC (SI =2-3.5). These findings demonstrate that T cell responses to Dsg3 are not only restricted by HLA-DR but also by HLA-DQ molecules. The DR11-restricted T cell response to Dsg3 is tuned by amino acid polymorphisms at positions 67, 71, and 86 of the β1-chain. Identification of the MHC II molecules that restrict Dsg3-specific T cell responses are crucial for the development of immunomodulatory peptides as a treatment for PV.

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SUPERANTIGENS PRESENTED BY HLA CLASS II' KERATINOCYTES OR PERIPHERAL BLOOD ACCESSORY CELLS INDUCE T CELL CUTANEOUS LYMPHOCYTE-ASSOCIATED ANTIGEN EXPRESSION VIA INTERLEUKIN-6.

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There is a growing body of evidence showing that superantigens are involved in the induction of T cell mediated dermatoses, such as psoriasis. Recently, we and others demonstrated that superantigens - presented by peripheral blood accessory cells - induce T cell expression of CLA via IL-6 and/or IL-12 production. We asked whether superantigens presented by human keratinocytes can induce T cell expression of CLA. Keratinocytes from 3 healthy donors (passage 4-5) were cultured in the presence or absence of IFNγ for 24h. Thereafter, highly purified T cells were cocultured with the keratinocytes for 4 days in the presence or absence of TSST-1. CLA expression by CD3+ cells cocultured in the presence of IFNγ stimulated keratinocytes showed a marked increase of CLA expression compared to keratinocytes cultured in the absence of IFNγ (mean T cell CLA expression at day 0: 13.9%; at day 4 of coculture in the absence of TSST-1: 12.7%; at day 4 of coculture in the presence of TSST-1: 42.5%). Addition of anti HLA-DR and -DQ mABs nearly totally blocked T cell CLA induction (T cell CLA expression at day 4 of coculture + TSST-1 - HLA class mAbs: 42.0%; at day 4 of coculture +TSST-1+HLA-class II mAbs: 17.3%). Furthermore, addition of IL-6 mAB partially inhibited T cell CLA induction in this coculture modell in the presence of TSST-1 (T cell CLA expression at day 4 of coculture+TSST-1 - anti IL-6mAbs: 42.0%; at day 4 of coculture+TSST-1+anti IL-6 mAbs: 26.1%). We conclude that IFNγ-stimulated keratinocytes present superantigens to T cells in an HLA class II bound manner. This results in increased CLA expression of these T cells, a reaction involving IL-6. Since bacteria residing in the skin are a common source for superantigens data generated with epidermal APCs such as keratinocytes might be of particular(patho-)physiological importance.

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INTERLEUKIN 12 PREVENTS AND REVERSES INDUCTION OF ACTIVE SUPPRESSION BY CUTANEOUS EXPOSURE TO SUPERANTIGEN.

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Cutaneous exposure to minute amounts of superantigens like staphylococcal enterotoxin B (SEB) leads to initial activation and subsequent long-lasting functional inhibition of SEB-reactive αβ T-cells in the exposed animal. In BALB/c mice such cells express as variable elements of their T-cell receptor β chain (Vβ) Vβ7 or 8. The aim of the present study was to determine whether the functional inhibition of Vβ7+ and Vβ8+ T-cells was only due to anergy or whether it was transferable, which cell types or soluble mediators might be involved and how it might be regulated.

BALB/c donor mice received intracutaneous injections of 50 ng SEB every other day over a period of two weeks at the lower abdomen. Then $50x10^6$ inguinal lymph node (LN) cells were transferred intravenously into naive syngeneic recipients, which were sacrificed two days later. Their spleen mononuclear cells (MNC) were assayed for their proliferative response to SEB among other stimuli by assessment of thymidine incorporation during the last 6 hours of 3 day cultures. Mice that have had received cells from SEB-treated donors exhibited a strongly decreased proliferative response to SEB, while spleen MNC from control mice that have had received cells from sham-treated donors proliferated normally. The inhibited proliferative response was due to an active mechanism, as the relative amount of transferred T cells did not exceed 5% of spleen MNC of recipients evidenced by marking of transferred cells prior to transfer. For significant suppression more than $5x10^6$ LN cells from SEB-treated donors were necessary. Suppression of proliferation was detectable as soon as 12 hours after transfer and declined after 7 days, but was still detectable after 3 weeks. Depletion of Vβ7+ and Vβ8+ or CD8+ or CD3+ cells (but not other Vβ+ or CD4+ or B220+ or I-A+ cells) abolished the capacity of inguinal LN cells from SEB-treated donors to mediate suppression. Treatment of the donors with murine recombinant interleukin 12 (IL-12) even after SEB-treatment abolished the capacity of inguinal LN cells from SEB-treated donors to transfer suppression. Sera from SEB-treated donors or supernatants from SEB-stimulated cell cultures were not able to transfer suppression of proliferation and did not contain amounts of transforming growth factor-ß1 or IL-10 detectable by ELISA.

These data indicate that cutaneous exposure to SEB induces suppressor cells which are Vβ7+ or Vβ8+ CD8+ T-cells and that active suppression can be prevented and reversed by IL-12 treatment in vivo. This may be of importance for the understanding and treatment of diseases associated with staphylococcal colonization and for chronic / recurrend staphylococcal infections.

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NORMAL HUMAN STRATUM CORNEUM CONTAINS AT LEAST FOUR T CELL STIMULATORY COMPONENTS WITH ANTIGENIC PROPERTIES.

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We have previously reported the presence in aqueous stratum corneum (SC) extracts of HLA-DR-dependent T-cell stimulatory material which may represent novel antigen. We have proposed that this is normally sequestered from the immune system but may induce pathogenic immune responses when released in disease and wounding. We now report further characterization of this material. Aqueous SC extracts were prepared from electric razor shavings and heel SC and shown to induce proliferation of autologous peripheral blood mononuclear cells (PBMC), from which T cell lines were prepared. Reversed phase (RP) HPLC purification revealed major T cell stimulatory activity eluting at 50% acetomitrile. RP-HPLC-purified SC extracts were subjected to chromatofocusing on a TSK-DEAE-5-PW column eluted with an ammonium acetate pH gradient. Proliferation assays of fractions with PBMC and T cell lines showed four stimulatory components with pI values varying between 9 and 4. RP-HPLC-purified T cell stimulatory material was also shown to be stable to denaturation and was thus subjected to denaturing Tris-Tricine SDS-PAGE with electroblotting onto nitrocellulose and PVDF membranes. Two mm strips of washed nitrocellulose were included in three-day proliferation assays with an SC-reactive T cell line and irradiated autologous PBMC as antigen presenting cells. This revealed at least three stimulatory components, the major one migrating at 9kDa. This corresponded on PVDF membrane blotting with a major Coomassie Blue-stained band, which was excised and subjected to automated microsequencing. A 19-residue N-terminal sequence identical with that of the cysteine protease inhibitor cystatin A, which has to our knowlege not previously been identified in human skin, was obtained. The immunostimulatory properties of authentic cystatin A are being investigated. These studies are therefore close to identifying what may be the first T cell dependent antigens in human skin.

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HUMAN SKIN GRAFTED ON SCID MICE AS A MODEL FOR STUDYING THE MIGRATION OF HUMAN T CELLS TO THE SKIN

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T cells migrate to the skin in various skin diseases, including cutaneous T cell lymphoma. In order to study skin migration of T cells in vivo we used a human severe combined immunodeficiency (SCID) mouse model. Buttock skin from healthy volunteers was grafted onto SCID mice. After 2 weeks, T cells were injected i.p. and subsequent cell infiltration of the skin graft characterized. Nickel-specific T cell lines derived from allergic individuals resulted in infiltration of the autologous graft with mononuclear cells that were significantly more numerous in mice receiving nickel in Vaseline applied to the skin graft than mice receiving Vaseline and T cells, mice receiving nickel in Vaseline but no T cells or mice receiving a nickel non reactive T cell clone and nickel in Vaseline. Analysis indicated that a large part of the infiltrate consisted of human T cells. When nickel-specific T cell lines were labelled with a intracellular fluorescent dye before i.p. injection, grafts challenged with nickel became infiltrated with fluorescent cells, strongly indicating that i.p. injected human T cells were migrating to the grafted skin. To give evidence for skin migration of T cells in an independent experimental system, HUT-78 cells that are derived from a patient with HTVL-1+ cutaneous T cell lymphoma and that express the Vβ23 clonotypic T cell receptor, were injected i.p. into mice with human skin grafts. Analysis of the T cell receptor repertoire in grafted skin showed that 10/31 mice injected with HUT-78 cells but none of 7 mice receiving grafts alone exhibited Vβ23 transcripts. Together, these results suggest strongly that nontransformed as well as some neoplastic human T cells can migrate to the skin in this human SCID model and that the increase of T cells in skin grafts is not only due to local expansion of T cells previously present in the graft. This human SCID model should therefore allow to study in vivo factors that control the migration of human T cells to the skin.

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CD44 VARIANT ISOFORMS IN SKIN TRANSPLANTATION. S.Seiter[1], S.Büchner[2], W.Tilgen[1], M.Zöller[2]. [1]Dpt of Dermatology, Univ of Heidelberg; [2]Dpt of Tumorprogression and Immune defense, German Cancer Res. Center Heidelberg; FRG.

One possibility to avoid allograft rejection is based on general immunosuppression. Due to the severe side effects, several regimen i.e. induction of tolerance by preimmunisation with donor cells under blockade of either costimulatory molecules on host cells or their ligands are currently being explored. Recently it has been described that blocking an adhesion molecule and its ligand results in acceptance of heart transplants without any immunosuppression. Knowing that the adhesion molecule CD44 is involved in lympocyte activation, too, we investigated whether blocking of CD44s or CD44v6 isoforms can interfere with acceptance of skin transplants. Compared to CD44s blockade of CD44v6 would be advantageous as CD44v6 is expressed by far less abundantly and its expression on lymphocytes is restricted to the activation period. When BDX rats received full-thickness (DAxBDX)F1 skin grafts together with intravenous injections (iv) of either anti-CD44s or anti-CD44v6 monoclonal antibodies (mAB) rejection was retarded by 4-7 days but none of the animals accepted the graft. Under low dose treatment with cyclosporin grafts were also rejected, albeit with delay. However combining low doses of cyclosporin and anti-CD44v6 mAB iv allowed for graft acceptance. Immunohistology of the graft and surrounding tissue during the post implantation period revealed reduced numbers of infiltrating lymphocytes and monocytes. Furthermore less cells were recovered from draining lymph nodes of these animals and limiting dilution analysis revealed that particularly the expansion of allospecific cytotoxic t cells was strongly reduced. Whether combining low dose cyclosporin with anti-CD44v6 mAB can induce long lasting tolerance is currently being explored by regrafting BDX rats with (BDXxDA) F1 versus third party allogenic skin.

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EXPRESSION AND REGULATION OF A NOVEL T CELL ACTIVATION MARKER (SLAM) ON LYMPHOCYTES FROM ATOPIC AND PSORIATIC SUBJECTS.

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Signaling Lymphocyte Activation Molecule (SLAM) is a newly described receptor involved in an alternative stimulatory pathway besides CD28/CD80/CD86 during T lymphocyte activation and expansion. We report that SLAM is expressed on freshly isolated peripheral $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes from atopic and psoriatic subjects. SLAM is upregulated on atopic $CD19^+$ B lymphocytes (52%), psoriatic $CD4^+$ and $CD8^+$ T lymphocytes (68% and 80%), compared with $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes from normal subjects (44%, 15% and 32%). Th1- and Th2-derived cytokines show differential effects on the expression of SLAM on lymphocytes from atopic and psoriatic subjects. A simplified summary of our findings showed that in the case of atopic subjects, Th1 cytokines (IL-2, IFN-γ, or TNF-α) upregulated the expression of SLAM on $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes. Th2 cytokines (IL-4, IL-10 and IL-13) selectively upregulated SLAM on $CD19^+$ B lymphocytes, but not on T lymphocytes. In the case of psoriatic subjects, Th1 cytokines downregulated the expression of SLAM on $CD4^+$ T lymphocytes, and upregulated SLAM on $CD8^+$ T lymphocytes and $CD19^+$ B lymphocytes. Th2 cytokines downregulated the expression of SLAM on $CD8^+$ T lymphocytes and upregulated those on $CD19^+$ B lymphocytes, whereas they have no effect on $CD4^+$ T lymphocytes. The importance of SLAM as an activation molecule on lymphocyte subpopulations needs to be further studied.

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CD8+ T CELLS WITH A TYPE 2 LYMPHOKINE PATTERN DO NOT PROTECT FROM VIRAL INFECTION

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CD4+ T cells with type 1 and type 2 lymphokine (lk) pattern have different, often opposing function in vitro and in vivo. This issue has not been studied in CD8+ T cells although CD8+ T cells can be induced to secrete either type of lk pattern. We thus studied the function of TCR transgenic CD8+ T cells with different lk patterns in vivo. The ab double transgenic (tg) TCR recognizes the lymphocytic choriomeningitis virus (LCMV) peptide 33-41. Protection against LCMV has previously shown to depend on CD8+ T cells and perforin. Protection can be conferred by CD8+ T cells expressing the tg TCR to the 33-41 peptide alone. We prepared tg T cells with a type 1, 2 and an unrestricted lk pattern in vitro using either plate-bound CD3 mAb or antigen presenting cells plus LCMV peptide with and without IL-4. These various CD8+ T cell subsets were injected i.v. into naive mice that were infected with LCMV 24 hours previously. Virus titers were determined in the spleen, liver and kidney after 4 days. Whereas T cells with a type 1 pattern conferred protection, the transfer of T cells with a type 2 or unrestricted pattern resulted in 2 (spleen) and 3 (liver and kidney) log higher virus titers than cells with a type 1 pattern. All three T cell subsets showed similar antigen-specific cytotoxicity in vitro at varying effector target ratios and constant peptide concentration. In addition, all 3 CD8+ effector cell types expressed similar levels of Fas ligand and they all induced antigen-specific DTH upon local transfer into foot pads. Thus, the paramount difference in antiviral protection of CD8+ effectors secreting a type 1 as opposed to CD8+ effectors with a type 2 and a nonrestricted lk pattern cannot be explained by the present findings.

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PERIPHERAL BLOOD LYMPHOCYTE SUBPOPULATIONS IN SEVERE APHTHOSIS. Renata Gorska, Jadwiga Dwilewicz-Trojaczek, Wieslaw Glinski. Dept of Mucous Membrane Diseases, Dept of Haematology, Dept of Dermatology, Warsaw School of Medicine, Warsaw, Poland

There is no agreement on the role of lymphocyte subpopulations in the pathomechanism of aphthous ulceration development. The purpose of this paper was to quantitate by flow cytometry peripheral blood lymphocyte subpopulations in patients with severe aphthosis, using a panel of monoclonal antibodies (prod. Dako A/S, Denmark) directed against various lymphocyte markers.

Twenty five patients with major and minor symptoms and 30 healthy subjects have been studied. There were no alterations in the percentage of T cells since only 5 patients with major aphthosis showed a significant decrease in CD2+3+4+ and 8+T cells. However, a slight increase in CD19+B cells has been found (10.3% vs 6.1% in normal controls; $p<0.05$). Mean percentage of CD25+ cells (8.8 +/-3.1%) was markedly ($p<0.01$) elevated in patients with aphthosis compared to healthy subjects (3.2 +/-2.9%), which is in favour of IL-2-driven lymphocyte stimulation. In addition, there were many more naive CD45RA+ cells neither bearing CD4 nor CD8 antigen as well as less memory CD45RO+ cells of suppressor/cytotoxic phenotype in the patients.

These data suggested that the shift in lymphocyte subpopulations and relapse of aphthous ulcerations might be related phenomena.

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LORATADINE DECREASES THE ACTIVATION OF SEVERAL T-LYMPHOCYTE SUBPOPULATIONS IN MIXED EPIDERMAL CELL-LYMPHOCYTE REACTION.

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Loratadine is a peripheral H1-receptor antagonist which is also active on chronic cutaneous inflammatory diseases. It has been shown that it decreases the ICAM-1 expression on human keratinocytes and Langerhans cells, but no study has evaluated its direct effect on effector lymphocytes. We have studied the effect of the addition of several concentrations of loratadine (0.25, 2.5, and 25 µmol/l) on lymphocyte proliferations in mixed lymphocyte reaction (MLR) and mixed epidermal cell-lymphocyte reaction (MECLR). The lymphocyte subpopulations have been studied at days 0 and 6 by flow cytometric analysis after four-color staining (fluorescein, phycoerythrin, ECD, tricolor). The following antigens have been studied: CD3, CD4, CD8, CD16, CD19, CD25, CD26, CD28, CD38, CD45RA, CD45RO, CD56, CD100, CDw101, HLA-DR and BY55. Results show that loratadine decreases in a dose-dependent manner the lymphocyte proliferation in MLR (maximal inhibition 74%) and MECLR (maximal inhibition 83%). Loratadine induces a decrease of the percentages of lymphoid cells expressing antigens induced by activation: HLA-DR (median decreasing from 31% to 20%), CD25 (decrease from 13% to 8%, mainly on the CD3+CD4+ subpopulation), CD28 (decrease from 44% to 36%) and CD38, marker of a subpopulation of activated T lymphocytes (decrease from 21% to 13%). Similarly, the expression of CD100, expressed on activated T lymphocytes, is decreased from 70% to 35%. In contrast, the CDw101 antigen is increased on the CD3+CD4+ and CD3+CD8+ populations. In conclusion, loratadine significantly decreases the activation markers of several subpopulations of T-lymphocytes in allogeneic mixed epidermal cell-lymphocyte reaction. These effects on the effector pathway of the immune response may explain the efficacy of this molecule on cutaneous inflammatory diseases.

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INHIBITION OF ANTI-CD40+IL-4 MEDIATED B CELL PROLIFERATION AND IgE SYNTHESIS BY RETINOIC ACID IN VITRO.

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Retinoids are well known regulators of growth and differentiation in a number of cell types including B lymphocytes. The aim of this study was to determine the role of retinoids (RA) in anti-CD40+ IL-4 mediated B cell activation. The effects of all-trans and 13-cis retinoic acid (10^{-5}-10^{-5}M RA) on anti-CD40 (1µg/ml)+ IL-4 (5ng/ml) mediated proliferation and immunoglobulin synthesis of human PBMC and B cells were evaluated in healthy donors. Proliferation of cells was measured by [H^3] incorporation and IgE, IgG and IgA were detected in the supernatants after 10 days of cell culture by ELISA techniques. CD40+ IL-4 mediated proliferation of PBMC was inhibited by both RA in a dose dependent manner with maximal inhibition of 60%±12% for all-trans RA and of 59%±8.5% for 13-cis RA (n=4). The same pattern of inhibition was seen in purified B cells. There was an associated even more marked inhibition of anti-CD40+IL-4 induced IgE-synthesis by both RA down to concentrations $>10^{-10}$M (all-trans RA 82%±3.8 and 13-cis RA 79%±8.2%, n=4) which were not affecting B cell proliferation. In contrast, IgA and IgG production was not altered. On the basis of preliminary data using receptor specific RA, these effects are primarily RA α-receptor mediated.

These results show that all-trans RA and its 13-cis isomer are capable to inhibit anti-CD40+IL-4 mediated PBMC- and B cell proliferation and specifically inhibit IgE production in vitro. The relevance of these results in vivo needs to be further explored.

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HUMAN NICKEL-SPECIFIC T CELL CLONES BELONG TO BOTH CD4+ AND CD8+ SUBSETS. C. Moulon, M. Fritz, J. Vollmer, and H.U. Weltzien, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany.

Allergic contact dermatitis is a skin manifestation of delayed-type hypersensitivity induced by haptens and mediated by T lymphocytes. In man, the relative contribution of CD4+ and CD8+ T cells in the disease is poorly studied and few human hapten-specific CD8+ T cell clones have been described so far. The purpose of our study was to investigate the existence of both CD4+ and CD8+ hapten-specific subsets derived from donors sensitized to heavy metal ions such as nickel. Hapten-specific T cell lines were produced by in vitro stimulation of peripheral blood mononuclear cells from four nickel-allergic donors with NiSO4. Phenotypical analysis of these T cell lines indicated a majority (80-90%) of CD4+ T cells expressing an αβ TcR. A panel of different T cell clones was then isolated. Although the majority of the T cell clones obtained belonged to the CD4+ subset, some CD8+ T cell clones were isolated. These clones proliferated and also displayed cytotoxic activity in response to autologous EBV-B cells in the presence of Ni salts. These antigen-specific responses were MHC-restricted and some of the cytotoxic T cell clones exhibited crossreactivity to palladium. Further studies will be required to determine the nature of the epitopes recognized by these metal-specific T cell clones. Furthermore, the characterization of CD8+ clones will hopefully lead to a better understanding of their precise role in the pathogenesis of nickel contact dermatitis.

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INDUCTION OF CD8+ EFFECTOR T CELLS SPECIFIC FOR A DEFINED SYNTHETIC MHC CLASS I-BINDING HAPTEN-PEPTIDE COMPLEX *IN VITRO* FROM NAIVE T CELLS.

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CD8+ T cells have been shown to be involved in hapten-induced contact hypersensitivity reactions. Many trinitrophenyl (TNP)-specific T cell clones and hybridomas derived from CD8+ T cells of mice sensitized to TNP recognize TNP covalently coupled to lysine on position 4 of MHC class I binding peptides in the H-2b haplotype. We studied the induction of such effector cells from naive T cells *in vitro*. Primary stimulation was carried out with skin-derived dendritic cells from C57/BL6 mice, syngeneic CD8+T cells, and a MHC class I binding peptide (sequence GGYK*FGGL) with a TNP substitution at the ε-aminogroup of lysine in position 4* (TNP-peptide). After 5 days, we restimulated the T-cell blasts with TNP-peptide, the nonsubstituted peptide and RMA cells to assess lymphokine production and measured cytotoxic activity by a 51Cr release assay. Primary cultures with TNP-peptide but not peptide resulted in effectors that released IL-2 and IFN-γ in response to TNP-peptide. Restimulation with RMA cells in the presence or absence of peptide did not result in lymphokine release. Similarly, TNP-peptide induced effectors exhibited hapten and dose-dependent specific cytotoxicity. When FACS-purified naive (CD44low) CD8+ T cells were used the same effector phenotype was found. Furthermore, the addition of IL-4 to the primary cultures containing naive CD8+ T cells resulted in hapten-specific effectors that released IL-4 in addition to IL-2 and IFN-γ. These results show that it is possible to induce *in vitro* lymphokine producing and cytotoxic CD8+ effector T cells specific for a defined hapten carrier complex from naive T cells. Our system may help to define the molecular events that lead to CD8+ effector T cell populations that mediate contact hypersensitivity and that may suppress it.

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A KEY ROLE OF γδ T CELLS IN DELAYED (1 DAY) SKIN INFLAMMATION AFTER HAPTEN APPLICATION TO NAIVE MICE

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Application of haptens to the skin of naive animals causes a delayed in time inflammatory response that peaks 24 hours after hapten treatment. We investigated whether this primary hapten-induced contact dermatitis (PHICD) may involve elements of the adaptive immune system. If this is the case then animals tolerized with hapten may exhibit altered PHICD. Thus, animals were tolerized by the i.v. route or by low doses of epicutaneous hapten. Application of a sensitizing dose of hapten to tolerant mice led to a decreased PHICD compared to naive animals using both tolerization models. Decreased PHICD was hapten-specific. While these findings can be interpreted to support a role of the adaptive immune system in PHICD other interpretations are possible. To further investigate a possible role of cellular elements of the adaptive immune system in PHICD, we used animals in which various cellular lines of the adaptive immune system are depleted or deficient. PHICD was preserved in naive NK-depleted mice, but was greatly diminished in naive SCID mice. These results exclude a role of NK cells in PHICD and indicate that the involved cellular element may be T and/or B cells, as both cell types require a recombinase-dependent event to function. To test the importance of T and B cells independently, PHICD was assessed in young naive athymic nude mice and in naive B-cell deficient mice. Both mouse types showed an intact inflammatory response to primary hapten application when compared to naive control mice. Thus, PHICD appears to depend on a thymus-independent non-NK non-B cell that is absent in SCID mice, leaving Thy1+CD3+CD5-TCRγδ as candidate. We therefore analyzed δ gene knock-out mice and found that PHICD did not occur in these animals. These results clearly indicate a key role of γδ T cells in PHICD.

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SENSITIZATION AND TOLERANCE TO METHACRYLATES

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Methacrylates (MA) are well-kown contact sensitizers in man; patch tests nearly always show polyvalent sensitizations. In this study the clinically most important allergens methyl MA (MMA), 2-hydroxyethyl MA (2-HEMA), 2-hydroxypropyl MA (2-HPMA) and ethylene glycol diMA (EGDMA) were investigated in guinea pigs (GP) for their sensitization and tolerance induction capacities and cross-reactivity patterns.

Inbred guinea pigs, 8-13 per group, were immunized by sc injections of 300μl of 1M MA solutions in FCA in both flanks, ears and neck. Control groups received FCA only. After 14 days open skin tests were performed with 50 μl of 50% MA solutions (for EGDMA 10%) in DMSO 40% in ethanol at the lateral sides with readings after 6, 24, 48 and 72 h. Tolerance was induced by oral application (3x) of 175μl pure MMA, followed by sensitization attempts for MMA and EGDMA.

Almost all immunized GP became strongly sensitized to MMA (26/26), 2-HEMA (7/8), 2-HPMA (11/12) and EGDMA (12/12). Cross-reactivity studies showed that MMA, 2-HEMA and 2-HPMA induced delayed hypersensitivity against all MA including EGDMA. In contrast, the strong sensitizer EGDMA hardly induced cross-reactivity to the other MA. Also, EGDMA-sensitization was not impaired in animals which had been rendered MMA-tolerant by previous feeding.

The results of these studies offer new insights in MA allergies and the tolerance findings may allow future therapy strategies in high risk trades.

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COMPARISON OF THE IN VITRO SENSITIZATION TEST WITH THE GUINEA PIG MAXIMIZATION TEST FOR CONTACT ALLERGENS.

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We reported an in vitro hapten-specific sensitization method using Pam212 cells (in vitro sensitization test) to identify potential of contact allergens previously. In the present study we assessed sensitizing potency of 8 allergens and 2 irritants and compared the results with that of guinea pig maximization test(GPMT). Monolayered-Pam212 cells were treated with chemicals, T cells and macrophages of BALB/c mice were cultured with chemical-treated Pam cells for 5 days. The T cells were separated, and then cultured with chemical-treated BALB/c spleen cells. Three days later [^{3}H]methyl thymidine incorporation were counted. Our assay detected extreme, strong and moderate sensitizers of GPMT, however, the results of moderate sensitizers showed a fluctuating one. Stimulation index was as follows; trinitrobenzene sulfonate 4.8, dinitrofluorobenzene 2.5, *p*-phenylenediamine 2.1, hydroxyethyl acrylate 4.8, imidazolidinyl urea 2.4, mercaptobenzothiazole 2.3, nickel sulfate 1.0, sodium lauryl sulfate 1.1 and methyl salicylate 1.2. With the single exception of nickel sulfate, non-sensitizers were not positive in our assay. The results support the view that our assay may provide a rapid and objective screening test.

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REGULATORY MECHANISM OF STEROID INDUCED AUGMENTATION OF CONTACT HYPERSENSITIVITY BY KERATINOCYTE DERIVED CYTOKINES.

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We have reported that long-term topical steroid augments contact hypersensitivity reaction in mice. Therefore, we studied the mechanism of down-regulation of this steroid induced augmentation of contact hypersensitivity reaction.

Balb/c mice were applied on the flank skin with 50 μl of Diflucortron solution in ethanol on alternate day for 9 times. On day 14 when mice received 7th applications of diflucortron, each mouse was sensitized with 0.5% DNFB on same site with diflucortron (flank) or opposite site(back). On day 19, one day after the last application, each mouse was challenged with 0.15% DNFB on the right pinna. Next we studied the mechanism of down-regulation of this response, by local injection of IL10, IL1 β receptor antagonist, extracts of keratinocyte or fibroblast cell line before challenge test.

The down-regulation of augmented skin reaction was observed by the pretreatment of IL1 β receptor antagonist or extracts of keratinocyte. Partial down-regulation was observed by IL10, or extracts of fibroblast.

Augmented contact hypersensitivity reaction by topical steroid was down-regulated by IL10, IL1 β receptor antagonist or various substance derived from keratinocyte or fibroblast. Topical steroid might modulate the production of these inhibitory molecules by the resident cells of the skin resulting in augmented skin reaction.

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Differential epidermal lymphocyte infiltration after irritant application.

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Irritant contact dermatitis (ICD) is a common occupational skin disease which shares histological features with allergic dermatitis, although its pathogenesis remains poorly understood. We investigated intracellular adhesion molecule 1 (ICAM-1) expression and lymphocyte infiltration in uninvolved skin and in early (1 and 6 hrs) and late (24 and 48 hrs) irritant reactions from 47 patients with chronic ICD. Irritants were 80% nonanoic acid (NA) or 5% sodium lauryl sulphate (SLS), titrated on volunteers to produce similar grades of erythema by 24 hours. Six µm frozen sections were stained for ICAM-1, CD3, CD4 and CD8. Numbers of positive epidermal lymphocytes were scored using the HOME microscope, and ICAM-1 assessed by light microscopy.

NA failed to induce epidermal ICAM-1 expression or lymphocyte infiltration, with normal numbers of lymphocytes in all biopsies (<4 cells/mm basement membrane). In contrast SLS induced significant focal epidermal ICAM-1 expression by 6 hrs($p<0.02$). In addition epidermal CD3+ve lymphocytes were seen by 24 hrs ($p<0.05$) and 48 hrs ($p<0.02$). At 24 hrs the ratio of CD4+ve /CD8+ve lymphocytes was 3:1, however by 48 hrs the numbers of CD8+ve lymphocytes had increased reducing the ratio to 2:1. **Hence only SLS induced either epidermal ICAM-1 expression or intraepidermal lymphocyte infiltration. CD4+ve cell recruitment was seen first (24hrs) and was followed at 48hrs by a CD8+ve infiltrate.**

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IMMUNE RESPONSES TO DNCB AND CARBAMAZEPINE ARE DEPENDENT ON INTERMEDIATE METABOLISM.

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In hypersensitivities to Carbamazepine (CBZ) and contact sensitivity to DNCB, demonstrable T cell reactivity in vitro is usually absent. We aimed to see if incubation of the suspected sensitiser with autologous macrophages would allow metabolism and generation of moieties able to elicit T cell proliferation. Peripheral blood mononuclear cells (PBMC) were isolated from 5 individuals previously sensitised with DNCB and 4 patients with CBZ-induced erythema multiforme. PBMC were placed in flat-bottomed wells to allow adherence of monocyte/macrophages (MM's). DNFB (less toxic than DNCB) or CBZ in different concentrations were added for 48h after which the supernatant (S/N) was removed and wells washed. Fresh PBMC were added to the adherent MM's or cultured with the S/N or native drug for 6 days. Proliferation, as ^{3}H-tdr incorporation, was expressed as stimulation ratio (SR). For both "antigens", native drug failed to elicit responses. For DNFB, S/N elicited only low responses: mean SR 1.15,(range 0.4 - 2.3). By contrast, PBMC cultured with DNFB-pulsed MM's responded strongly; mean SR 7.8 (range 4.6 - 11.8). For CBZ it was interesting to find that the S/N induced the stronger responses; mean SR 9.6 (range 3.3 - 140), whereas stimulation by adherent MM's was modest; mean SR 1.4 (range 1.2 - 2.8). Since DNCB is metabolised by glutathione s-transferase (GST) in vitro, ethacrynic acid, an inhibitor of GST, was added during the 48 hour pulse. Indeed, the response at 100µM DNFB was inhibited by 71% (SE 8) ($P<0.01$). CBZ undergoes first phase metabolism by P450 enzymes; addition of clotrimazole, an inhibitor of P450, reduced the response by 56% in the 1 subject tested. Our data indicate that DNCB, regarded as a hapten and thought to become immunogenic by virtue of strong reactivity with proteins, actually requires metabolic activation by GST. For CBZ this has long been suspected and the present method will allow manipulation and identification of the pathways involved.

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Studies on the mechanism of thimerosal sensitivity.

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Patch tests positivity to thimerosal have been subject of numerous studies but the results have pointed out several discordance. The nature of the reactions, the clinical relevance and the part of the molecule representing the elicitor have been not completely defined. To a better understanding of mechanisms underlining the thimerosal reactions, and to define molecular mediators or targets of the metal action, we have studied in 30 subjects patch tests positive to only thimerosal (0.1% pet): a) the sensitivity to other organomercurial compounds namely mersalyl acid and p-aminophenyl-mercuric acetate, b) the positivity to thiosalicylic acid; c) the rate of decomposition of thimerosal in aqueous solutions by gas chromatography mass spectrometry methods (GC-MS). Moreover, since it has been reported that Hg ions can bind zinc- or SH-containing proteins, the patients were patch re-tested to Thimerosal 0.05% (aq) and to mixed solutions containing thimerosal (0.05%) plus $ZnCl_2$ or $ZnSO_4$ 0.15%. In vitro the capability of SH-containing polypetides to combine with the Hg moiety of the molecule was evaluated by HPLC and spectrophotometric methods. All the patients gave positive reactions to only thimerosal. Both Zn salts were capable of inhibiting the patch test reactions to thimerosal. GC-MS and HPLC analyses revealed that thimerosal can easily release the mercury moieties in slight acidic solution (pH 5.5) and that the generated compound can combine with SH-containing polypeptides. Our results suggest that: 1) the mercury moiety is the responsible of the patch test reactions to thimerosal; 2) the mechanism of the dangerous effect of Hg may be due to its oxidation and subsequent binding to Zn or SH-containing structure.

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TWO CASES OF PATHWAY-DEPENDENT INDUCTION OF TYPE-IV HYPERSENSITIVITY REACTIONS ILLUSTRATE SPECIALIZATION OF DIFFERENT IMMUNOLOGICAL COMPARTMENTS.

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Limitation of HLA class II expression to certain cell types, promiscuity of peptide-HLA interactions and lymphocyte homing contribute to the economical function of the specific immune system providing a basis for organ-/compartment-specific immune responses. We here report two cases exhibiting type-IV hypersensitivity reactions depending on the pathway of the respective antigen best explained in this context: Patient 1 has a history of erythematous plaque reactions towards s.c. applied heparins at the sites of administration. Patch test, i.c. tests and s.c. provocation yielded positive results after 3-6 days for heparins and heparinoids; microscopy revealed an ekzematous pattern. I.v. infusion with two positively tested drugs was tolerated. Patient 2 developed type-IV reactions upon application of an estrogen containing transdermal therapeutic system. Patch testing documented the active drug estradiol itself to be the causative agent. Subsequent use of this drug in an oral formulation was tolerated. These cases are both of clinical importance (case 1: cross reactivity of heparins and heparinoids initially developed to substitue allergy inducing heparins; case 2: allergy to an endogenous hormone) and immunologically meaningful since they illustrate a distinct state of readiness to respond to the same immunogen in a compartment-depending manner.

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EORTC CLASSIFICATION FOR PRIMARY CUTANEOUS LYMPHOMAS

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Primary cutaneous lymphomas represent a heterogeneous group of T- and B-cell lymphomas, that show considerable variation in histology, phenotype and prognosis. Recently, the EORTC Cutaneous Lymphoma Project Group has reached concensus on a new classification for this group of disaeses. In contrast to other classification schemes for non-Hodgkin lymphomas, the EORTC classification for Cutaneous Lymphomas is not only based on histological and immunophenotypical, but also on clinical criteria. Therefore, it includes a list of disease entities, that have a well-defined clinical presentation and clinical behaviour. For that reason, secondary cutaneous lymphomas as well as cutaneous lymphomas arising in immunocompromised patients are excluded from this classification. In addition, it contains a number of provisional entities, which may display characteristic histologic features, but are clinically not yet well-defined. These provisinal entites account for less than 5% of all primary cutaneous lymphomas.

The basic principles of this new classification, the characteristic clinical and histological features as well as the estimated 5-year-survival data of over 600 patients with different types of cutaneous lymphomas registered at the Dutch Cutaneous Lymphoma Working Group, validating the clinical significance of this classification, are presented.

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GENETIC CHANGES IN CUTANEOUS T-CELL LYMPHOMA AS DETECTED WITH COMPARATIVE GENOMIC HYBRIDIZATION.

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Our aim was to identify changes in DNA-copy number in blood lymphocytes of CTCL patients. The DNA of 4 patients with mycosis fungoides and 5 patients with Sézary syndrome was studied using comparative genomic hybridization (CGH). The method detects losses and gains of DNA sequences and localizes them in their respective chromosomal regions. The sensitivity of CGH for amplified DNA is about 2 MB (5- to 7-fold amplifications of oncogenes) and for deletions 10-20 MB in cells with a diploid chromosomal modal number. The method requires at least about 50% of similarly aberrant cells.

All 5 patients with Sézary syndrome and 1 with mycosis fungoides had gains or losses affecting 1 to 6 chromosomes. Most of them concerned gains of whole arms or whole chromosomes. Chromosome 19 or 19q gain was detected in 5 patients, and chromosome 22 or 22q gain in 4 patients. Chromosome 17q gain was observed in 3 and 17p loss in one patient.

This is the first report showing gains or losses in DNA copy number in specific chromosomes in CTCL. The changes may highlight location of genes with a role in the development or progression of CTCL. CGH is especially valuable in the study of CTCL, because it does not require dividing cells, often difficult to obtain in this disease.

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P53 AND BCL2 EXPRESSION DO NOT CORRELATE WITH PROGNOSIS IN PRIMARY CUTANEOUS LARGE T-CELL LYMPHOMAS.

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Overexpression of p53 has been reported in primary cutaneous large T-cell lymphomas (PCLTCL) and has been associated with tumor transformation in mycosis fungoides. The objective of this study was to investigate the expression of p53 as well as bcl2 protein on PCLTCL and to correlate the results with follow up data and proliferative activity. 27 cases of PCLTCL (CD30+ n=19, CD30- n=8) were selected from the national registry of the Dutch Cutaneous Lymphoma Working Group. Immunohistochemistry was performed on paraffin sections using 2 antibodies against p53 protein (DO-7 and BP 53-12-1), DAKO bcl2, 124 for bcl2 detection and MIB-1 as a proliferation marker.

Overexpression of p53 protein was found in 6 of 19 (31%) CD30+ and 4 of 8 (50%) CD30- PCLTCL. Bcl2 protein was found in 7 of 19 (37%) CD30+ and in only 1 of 8 (12%) CD30- PCLTCL. No correlation between p53 or bcl2 expression was found neither in the whole group nor within the CD30+ or CD30- group. The presence of p53 and bcl2 did not correlate with the proliferating cell fractions.

These results confirm that p53 expression is common in PCLTCL. A relationship between p53 or bcl2 expression and prognosis or with proliferative activity was not found.

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Differential balance between p21$^{waf-1/cip-1}$ and p53 in bcl-2+ and bcl-2- cutaneous T Cells Lymphomas.

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Recent "in vitro" models have demonstrated that bcl-2 influences cell cycle machinery by down-regulate p21. This cyclin inhibitor, previously reputed to be activated only by p53, has also been observed at high concentration during cellular terminal differentiation and senescence. In order to convalidate in vitro data, a panel of 15 cases of intermediate and high proliferation index cutaneous T Cells Lymphomas has been studied, by use specific monoclonal antibodies and APAAP technique, for the expression of p21, p53, MDM2, pRB-1 and Mib-1. Subsequently p21/p53 ratio (p21/p53) was calculated for homogeneous group of pathologies (group a = 5 cases of bcl-2-, CD30+ Large Cell Lymphoma; group b = 3 cases of bcl-2+, CD30+ Large Cell Lymphoma; group c 5 cases of bcl-2+, CD30- medium/large Pleomorphic T Cells Lymphoma; group d = 2 cases of bcl-2+,CD30- small/medium Pleomorphic T Cell lymphoma). Results obtained for each group are as follow. Group a: 55%<Mib-1<72%, 30%<p53<45%, 30%<p21<60%, 5%<MDM-2<15%, Rb1= 0%, p21/p53 mean value (mv) 0,8. Group b: 30%<Mib-1<45%, 18%<p53<40%, 3%<p21<10%, MDM-2= 0%, Rb1= 0%, p21/p53 mv 0,25. Group c: 45%<Mib-1<60%, 10%<p53<40%, 3%<p21<10%, MDM-2 = 0%, Rb1= 80% (2 out of 5 cases), p21/p53 mv 0,2. Group d: 7%<Mib-1<15%, 8%<p53<15%, 1%<p21<5%, MDM-2 = 0%, Rb1= 0%, p21/p53 mv 0,3. Our results indicate an high p21/p53 ratio (0,8) in bcl-2- disorders and a low p21/p53 ratio (0,2-0,3) in bcl-2+ disorders. In conclusion, as suggested by the experimental model, in vivo data substain and validate a low tendency to terminal differentiation and senescence of bcl-2 positive Cutaneous T Cells Lymphomas.

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DIFFERENCES IN THE EXPRESSION OF BCL-2 PROTEIN AND ADHESION MOLECULES BETWEEN PROGNOSTICALLY DIFFERENT GROUPS OF PRIMARY CUTANEOUS LARGE B-CELL LYMPHOMAS.

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Primary cutaneous follicle center cell lymphomas (PCFCCL) presenting on the head or trunk represent a distinct disease entity with an excellent prognosis (5-year-survival > 95%). Morphologically similar lymphomas can also present on the lower legs. These patients develop more often recurrent and extracutaneous disease, and their prognosis is less favorable (5-year-survival 58%). The mechanisms underlying this different clinical behavior in these two groups of PCFCCL arising at different sites are as yet unknown. In this study PCFCCL presenting on head and trunk (n=13) or presenting on the legs (n=7) were investigated for the expression of bcl-2 protein. Since bcl-2 overexpression may result from the t(14;18), the presence of this translocation was studied with a PCR technique using mbr and mcr primers. In addition, the expression of a selected panel of adhesion molecules, including CD54/ICAM-1, CD11a/LFA-1 and CD49d was studied by immunohistochemistry. Bcl-2 protein was detected in 7/7 PCFCCL on the legs, but not in any of 13 PCFCCL on head and trunk. Bcl-2 expression was not associated with t(14,18). PCFCCL on head and trunk expressed ICAM-1 and LFA-1 in 8/10 and 5/10 cases, respectively, but no CD49d. In contrast PCFCCL on the legs did express ICAM-1 or LFA-1 in only 2/6 and 1/6 cases, respectively, but VLA-4 in 4 of 6 cases. Four of 5 lymphoma that were negative for both ICAM-1 and LFA-1 died of lymphoma; in contrast all cases that expressed ICAM-1 and/or LFA-1 are alive. These results suggest that differences in the expression of bcl-2 protein and adhesion molecules may contribute to the different behaviour of these two groups of PCFCCL.

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CD7 EXPRESSION ON SEZARY CELLS IDENTIFIES A SUBGROUP OF SEZARY SYNDROME PATIENTS WITH BETTER PROGNOSIS.

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Prognostic implications of PBL immunophenotyping in Sézary syndrome (SS) are not well delineated. The aim of this study was to evaluate circulating Sézary cell (SC) phenotype and ascertain if PBL immunophenotyping can provide prognostic information in SS. Cytofluorymetric PBL analysis was performed at diagnosis in 49 SS patients (5-yr survival: 42.9%). A T-helper memory phenotype was found in 41 patients (83.7%). In 3 cases, SC showed unusual phenotypes (CD4+CD8+, CD4-CD8- and CD2-CD4+CD8-CD57+, respectively), in 5 lacked CD2 expression. SC were predominantly CD7 positive in 20 patients (40.8%). CD26 was absent in all cases on SC. Survival univariate analysis showed that a CD4/CD8 ratio higher than 10, an absolute number of CD4+ cells more than 2,000 mm^3 and an absolute number of CD8+ cells less than 300 mm^3, were associated with unfavourable prognosis. Furthermore, patients with CD7- SC had a significantly worse prognosis than those with CD7+ cells. The independent predictors selected by proportional hazard regression were CD4/CD8 ratio, which increases the risk by 12 and absolute number of circulating CD4+ cells, whereas CD7 expression was of borderline significance. However, the predictive value of these factors was not confirmed when haematological parameters (including absolute number of circulating SC and presence of large SC) were included in the model.

In conclusion, our results confirm that circulating SC have a T helper memory CD26- phenotype; CD7 negativity is not a constant SC feature, demonstrating SS immunological heterogeneity. Immunophenotype prognostic relevance is correlated with SC count; however, CD7 positive SC may identify a subgroup of patients with better prognosis.

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Phenotypical grading of primary Cutaneous T Cell Lymphomas.

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A relevant prognostic role has been assigned to CD30 in recent classification on Cutaneous T Cells Lymphomas (CTCL). To solve the issue concerning the prognostic impact of several phenotypical markers, a retrospective study was conducted on a broad and heterogeneous group of 47 CTCL constituted of 15 cases of Mycosis Fungoides, 3 cases of Sezary's Syndrome, 10 cases of CD30- Pleomorphic T Cells Lymphoma, 6 cases of Lymphomatoid Papulosis and 13 cases of CD30+ Large Cells lymphoma. The cases have been evaluated for the phenotypical expression of bcl-2, CD2, CD3, CD4, CD5, CD7, CD8, CD25, CD30, CD95, Mib-1, and EMA, relating then the homogeneous phenotypical groups obtained to clinical outcome. Our results suggest that only specific antibodies like bcl-2, CD3, CD4, CD8, CD30, CD95, Mib-1 and EMA are able to fournish stringent prognostic informations. Survival curves for homogeneous phenotypical pathologies have convalidated the hypothesis. In conclusion we propose a phenotypical gradind (pG) for CTCL: pG1 (good prognosis, well biologically controlled lymphoprolifetrative disordes with high proliferation index) typical of bcl-2-, CD3+/-, CD4+ or CD8+, CD30+, CD95+, EMA+/- large cells CTCL, pG2 (disorders with low proliferation index and an intermediate risk of blastic transformation) for bcl-2+, CD3+, CD4+, CD30-, CD95-, EMA- small cells CTCL and pG3 (worse prognosis, disordes with an high proliferation index and an high risk of blastic transformation) for bcl-2+, CD3+, CD8+, CD30- , CD95- and EMA+ small and large cells CTCL.

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NUCLEOPHOSMIN (NPM) GENE REARRANGEMENTS IN CD 30-POSITIVE CUTANEOUS LYMPHOMAS

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Rearrangements of chromosome 5 at band q35 are often associated with CD30-positive lymphomas. Rearrangements may result in (3;5)(q12:q35) or (2;5)(p23:q35) translocations, the latter leading to formation of a chimeric protein of the cell proliferation-related nucleolar phosphoprotein NPM with the tyrosine-kinase receptor-homologous ALK gene.

By Southern blotting, we have analyzed NPM gene rearrangement in 10 CD30-positive cutaneous lymphomas including 8 anaplastic large cell lymphomas (ALCL) and 2 lymphomatoid papuloses (LyP) of the skin.

Using a genomic DNA probe complementary to the 5'untranslated NPM sequence, we detected rearrangements of the NPM gene in 3 cases of ALCL and in both cases of LyP. Our results suggest the presence of NPM gene rearrangements in a significant subset of CD30- positive cutaneous lymphomas and may identify subgroups in CD 30-positive lymphomas that may relate in terms of molecular features.

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Th2 CYTOKINE EXPRESSION IS UNCOMMON IN CD30-POSITIVE AND CD30-NEGATIVE PRIMARY CUTANEOUS T-LARGE CELL LYMPHOMAS (PCTLCL).

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Recent studies demonstrating IL-4, IL-5 and IL-10 in Sézary's syndrome (SS) and tumor stage mycosis fungoides (MF), have led to the concept that cutaneous T-cell lymphomas (CTCL) are of the Th2 subtype. The presence of mRNA for IL-2 and IFNγ in early MF has been attributed to reactive tumor infiltrating T-cells. However, CTCL other than MF and SS have not been studied thus far. In the present study skin biopsies from 9 CD30+ and 5 CD30- PCTLCL were studied for the expression of Th1 and Th2 cytokines using reverse-transcriptase PCR. Skin biopsies from SS (n=5), MF (n=5), psoriasis (n=3) and lichen planus (n=2) were used as controls. Consistent with prior studies, a Th2 profile was detected in SS and tumor stage MF, whereas a Th1 profile was found in patch stage MF, psoriasis and lichen planus. However, in CD30- PCLTCL no IL-4 and in only 1 of 5 cases both IL-5 and IL-10 were found. In 9 CD30+ PCTLCL IL-4 and IL-10 mRNA were detected in only 2 and 5 of 9 cases, whereas IL-5 was not detected. IL-2 and IFNγ mRNA were detected in 8 and 12 of 14 PCTLCL, respectively. Separation of CD30+ tumor cells using the MiniMACS method in a patient with a CD30+ PCTLCL showed that mRNA for IL-2 and IFNγ was only present in the fraction with tumor infiltrating T-cells (<5% of the infiltrate). The results of this study indicate that both CD30+ and CD30- PCTLCL uncommonly express Th2 cytokines, which underscores the differences in the immunopathogenesis of the different types of CTCL.

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Cytokines and cytokine receptors pattern in primary cutaneous CD30+ Large Cells Lymphomas.

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Cytokines play a pivotal role in the physiological control of the immunitary system through stimulatory and inibitory signals. T lymphocytes, during activation phase, down-regulate bcl-2 protein acquiring several cytokine receptorial molecules on their surface. An almost constitutive expression of some receptorial cytokine molecules is observed in CD30+ Large Cells T Cells Lymphomas (LCL). In order to evaluate soluble cytokines production and cytokine receptors pattern, 16 cases of LCL have been studied, by use several monoclonal antibodies and an APAAP technique, for the expression of IL-1α, CD25, CD30L, IL-4, IL-6, IL-8, IFN-γ, IL-3αr, IL-4r, IL-6r, gp130, IL-7r, IL-8r, TNF-r1/r2, TGFβ-r, 4-1BB, OX-40, and HECA-452 (IL-1r, IL-4r, IL-7r and CD30L are a gift of Dr. Armitage, Immunex, Seattle, U.S.A). Positive results were observed for: HECA-452 (10/16 cases), IL-4r (8/16), CD25 (6/16), TNF-r2 (8/16), IL-3αr (5/16), TGFβ-r (5/16), , 4-1BB (5/16), IL-6r (3/16), gp130 (3/16), IL-1r (3/16), OX-40 (1/16), and IL-8 (5/16). No detectable signal was observed for CD30L, IL-7r, IL-8r, IFN-γ, IL-1, IL-4, IL-6, TNF-r1. Only two cases displayed a double expression of IL-4r and CD25. The data point attention to a lack of specific autocrine mechanisms in LCL and that they are concerning cytokine receptors, an heterogeneous group of pathologies, being TNF-r2 and IL-4r the most expressed ones. Though it is not possible to affirm a Th1 or Th2 derivation of LCL T lymphocytes, Il-4r expression, observed in 50% of analyzed cases, suggests that Th2 accessory cells display a key role in the paracrine control of these lymphoproliferative disorders.

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THE ACCESSORY FACTOR (AF-)-1 (INTERFERON γ RECEPTOR β CHAIN) IS A MARKER FOR CIRCULATING SÉZARY SYNDROME'S T HELPER 2 T-CELL CLONES IN VIVO

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Sézary Syndrome (SS) is a leukemic low-grade cutaneous T-cell lymphoma. Using antibodies against the variable region of the T-cell receptor TCR (Va/b) we have identified four predominant T-cell clones (2 Vbeta 8+ clones, 1 Vbeta 5.1+, 1 aV2(a)+) in SS patients' peripheral blood mononuclear cells (PBMC). Their phenotype was CD3+, CD4+, CD5+, CD45RO+. Clonal T-cells were purified and cytokine transcription and secretion was analysed by RT-PCR followed by hybridisation with biotinylated probes and ELISAs. The IL-10 PCR product was cloned and sequenced and found to be identical to the published cDNA sequence. The presence of AF-1 encoding mRNA was assessed by RT-PCR and immunostaining using serum of rabbits immunized with the extracellular domaine of a recombinant human AF-1 protein followed by APAAP staining. Clonal T-cells transcribe and secret mainly T helper 2 cytokines (Interleukin-10, -5, -13). mRNA from purified SS clones but not from mRNA of SS total PBMC was positive for AF-1 in an agarose gel and/or after hybridisation. AF-1 transcription was associated with membrane-bound immunoreactivity for AF-1 in SS clones.
SS derived T-cell clones display T helper 2 cytokines and show overexpression of AF-1 suggesting that AF-1 is a marker for circulating Sézary cells and possibly other T helper 2 cells in vivo.

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CHARACTERIZATION OF PERIPHERAL BLOOD LYMPHOCYTES IN CUTANEOUS T CELL LYMPHOMA (CTCL).

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CTCL are characterized by a hyperproliferation of malignant T-helper cells in the skin. However, recent results suggest that even in early stages of the disease malignant cells are also present in the blood. Moreover several phenomens indicate general immune abnormalities. It has been shown that tumor-reactive T cells are present in CTCL skin lesions and lower numbers of these cells correlate with worse prognosis. We wondered if a systemic anti-tumor response is demonstrable in peripheral blood of CTCL patients. Using flowcytometry blood samples from 37 CTCL patients of different stages were investigated in comparison to those from psoriasis (n=15), atopic dermatitis (n=11), and healthy volunteers (n=15). No significant differences were found for total T and B cell numbers. However, in CTCL patients considerable T cell activation, mainly characterized by increased percentages of HLA-DR+, TfR+ and LFA-1 high-expressing T lymphocytes ($p<0.005$) was demonstrated. Moreover, NK cell numbers were elevated ($p<0,05$). Wondering which T cell subpopulation is activated we showed that the CD8+ T cells is the highest activated subset. These cells are suspected to be cytotoxic T lymphocytes. Interestingly, a stage-dependent decrease in T lymphocyte activation was found in the course of CTCL. So in progressive tumor stages lower percentages of activated T cells were found compared with early stages ($p<0.05$). Marked T cell activation and elevation of NK cell numbers, especially in early CTCL stages, exclude the lack of anti-tumor response in CTCL. However, the stage-dependent decrease of these activation signs suggests the development of a lack in tumor surveillance in the progression of CTCL.

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LASER DISSECTION OF SINGLE LYMPHOCYTES AND POLYMERASE CHAIN REACTION ANALYSIS IN THE DIAGNOSIS OF CUTANEOUS LYMPHOMAS. Lorenzo Cerroni, Günter Minkus,* Barbara Pütz, Heinz Höfler,* Helmut Kerl. Department of Dermatology, University of Graz, Austria, and * Institute of Pathology, Technical University of Munich, Germany.

Interpretation of molecular analyses of cutaneous lymphoid infiltrates may be difficult because a heterogeneous group of cells is usually present within the neoplasms. Extraction of DNA from tissue sections does not provide exact informations about which cell population has been analyzed. We present a laser-microscope system that allows selective molecular analysis of single cells in cases of cutaneous lymphoma. A UV-laser microscope system (P.A.L.M.©, Wolfratshausen, Germany) was used to isolate particular populations of cells from a routinely-processed specimen of cutaneous follicular lymphoid proliferation. Shooting the laser beam a circle was cut around a target germinal center in order to separate it from neighboring tissues and to isolate a pure population of germinal center cells. Isolated cells were scraped off with a micromanipulator and placed in a proteinase-K solution. DNA was extracted and amplified by the PCR technic. Analysis of immunoglobulin J_H gene rearrangement showed a distinct monoclonal band. In a second phase, using the same procedure in the same specimen, mantle zone cells around a germinal center were isolated for PCR analysis of immunoglobulin J_H gene rearrangement. In this population, no clonality could be detected.

This new technic allows the selective elimination of undesired cells and tissue from cutaneous neoplasms. By vaporization of tissues with laser-beam energy a contamination-free sample is obtained. Analysis of isolated single cells in our case demonstrated a clonal rearrangement derived from germinal center cells and not from other B cells in the specimen, confirming the diagnosis of cutaneous follicle center lymphoma. The method just described has exciting implications for dermatology, allowing precise correlation of morphologic features with findings by molecular genetics.

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SKIN-INFILTRATING LYMPHOCYTE FLOW-CYTOMETRIC IMMUNOPHENOTYPING: AUTOMATED MECHANICAL BIOPSY DISAGGREGATION AND CD45 GATING. M.Novelli, M.T. Fierro, F. Lisa, A. De Matteis, P. Quaglino, P. Savoia, A. Verrone, I. Cambieri, M.G. Bernengo, 1ST Dermatologic Clinic, University of Turin, Italy.

Histological differential diagnosis between cutaneous reactive and malignant lymphoid infiltrates may be difficult. Immunohistochemistry can not identify subpopulations defined by expression of more than one antigen. Flow-cytometric analysis of disaggregated tissue suspensions can be performed only in presence of massive lymphoid infiltrates. Recently, an automated mechanical disaggregation device (Medimachine, Dakopatts, Denmark) has been introduced for cell isolation by tissue specimens. Sixty skin biopsies from different cutaneous diseases were analyzed using this method and compared with routinary immunohistochemistry. Biopsy fragments were dissociated using the Medimachine and three color flow-cytometry (CD45PerCP, MoAbFITC and MoAbPE) was performed on cellular suspension with CD45PerCP gating. Although only preliminary data are available, some interesting findings come to light. Firstly, CD45 gating increases lymphocyte isolation purity (95%-100% vs 60%-80%). Moreover, when compared with methods based on cultured lymphocytes from skin biopsy, automated mechanical disaggregation is easier and more reproducible, allowing a more correct qualitative and quantitative analysis of lymphocyte subsets. Therefore, this method can have diagnostic relevance in the differential diagnosis between pseudolymphoma and B lymphoma (cytofluorimetry easily detects a monoclonal component) and between early-stage cutaneous T-cell lymphoma (CTCL) and benign dermatitis, and in the detection of minimal residual disease in B cell lymphoma. Moreover, cutaneous lymphocyte subsets CTCL-related (CD4+CD7-, CD4+CD26-), or functionally involved in skin homing (CD4+CLA+, CD4+CD62L+) can be compared with their circulating counterpart, giving new insights in CTCL pathogenesis and cell trafficking network.

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The VH gene repertoire expressed in B cells infiltrating plaque lesions of patients with Mycosis fungoides. Nicole Förste, Sigbert Jahn, Wolfram Sterry
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It is well established that B lymphocytes (mostly plasma cells) appear within cell infiltrates contained in plaque lesions of patients with Mycosis fungoides. The question may be raised if a local B cell maturation and differentiation takes place under the influence of T cells belonging to the malignant clone. Processes of B cell selection and expansion are reflected by alterations of the gene segments encoding the variable portion of the immunoglobulin molecule. Therefore, the repertoire of the respective VH genes expressed by B cells in Mycosis fungoides was studied by RT PCR using sequence-specific primers: at the 5' end primers homologous to the different VH gene families, at the 3' end primers to CH1 domains of different heavy chain genes (my, gamma, epsilon). Using this approach, the VH gene transcripts contained in genes encoding the production of immunoglobulins of different isotypes can be analysed.

We show data supporting that the B cells infiltrating Mycosis fungoides lesions use a polyclonal VH gene repertoire, since a high amount of different VH gene family members was detected within the gene transcripts. There were no clonal relationships detected due to an individual VH-D-JH transcript in each clone sequenced. Both mutated and un-mutated VH genes were found to be expressed, supporting the hypothesis that memory as well as virgin B cells infiltrate the Mycosis fungoides lesion.

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GENOTYPING OF CUTANEOUS LYMPHOMAS BY PCR AND FRAGMENT ANALYSIS - DIGNITY OF OLIGOCLONAL FRAGMENT PROFILES. M. Muche, U. Scheller, A. Lukowsky, W. Sterry, Department of Dermatology, Medical Faculty (Charité), Humboldt-University Berlin, FRG

Genotyping by PCR for rearrangements of the TCR gamma genes followed by high-resolution electrophoresis (temperature gradient gel electrophoresis -TGGE) has been established recently in the diagnosis of CTCL. As a new approach the fragment analysis (FragA) allows for objective depiction of the PCR product but separates them only by their lengths. To evaluate this technique DNA of 45 CTCL biopsies suspected to be dominated by a T cell clone were analysed by PCR and subsequently separated by both TGGE and FragA.

In 32 cases the results concurred, 8 were only monoclonally by FragA, 1 was only monoclonally by TGGE. Four cases (2 monoclonal, 2 polyclonal by TGGE) showed a distinct pattern of the fragment profile with multiple peaks. These so-called oligoclonal cases appeared especially in early mycosis fungoides. To confirm whether this pattern represents the infiltrating T cells or is the substrate of an oligoclonal genesis of CTCL 12 cases of dermatitis were further analysed. In 11 cases a polyclonal profile was found by both separation techniques whereas one case was monoclonal by FragA. Although oligoclonal expansion of T cells could be expected theoretically in benign inflammation oligoclonal fragment profiles were not seen.

Our results demonstrate that FragA is a reliable and objective tool for detection of dominant clones in skin biopsies which is more sensitive than TGGE. Oligoclonal fragment profiles might be a special feature of malignant disease. They could indicate a step-wise genesis of CTCL from polyclonal via oligoclonal to monoclonal T cell proliferation.

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IL-10 mRNA OVEREXPRESSION IN CUTANEOUS T CELL LYMPHOMA. K. Asadullah, A. Haeußler, W.D. Döcke, H.D. Volk, W. Sterry Depts of Dermatology and Immunology, University Hospital Charité, Berlin, Germany.

Cytokines are considered to be of major importance in the pathogenesis of CTCL. There are partly conflicting data regarding the local cytokine pattern in Mycosis fungoides(MF). Recent studies suggested a shift from type 1 to type 2 cytokine pattern since interleukin(IL)-4 and IL-5 mRNA, were more frequently detected in lesions of advanced stages. Another group described a type 1 phenotype in MF lesions. However, all studies of cytokine mRNA expression in MF done before did not use quantitative methods and therefore could give only information whether a cytokine was detectable but not about the level of expression. To get closer insight into the development of cytokine pattern during tumor progression we used semiquantitative RT-PCR to investigate cytokine mRNA expression in MF skin lesions of different stages. Biopsies from patients with patch (n=11), plaque (n=6), and tumor (n=3) stage MF were investigated in comparison to those from patients with pleomorphic T cell lymphoma (n=5), psoriasis (n=7), atopic dermatitis (n=5), and from healthy skin (n=8). MF progression was associated with significantly higher IL-10 and lower IFNγ expression. Interestingly, the stage-dependent increase of IL-10 mRNA expression was also evident in paired samples from individual patients. However, in contrast to pleomorphic T cell lymphoma, typical Th2 cells seem not to be the source of increasing IL-10 expression in advanced MF, because, stage-independent, IL-4 mRNA was rarely detectable, suggesting contribution of non-lymphoid cells in local IL-10 production. The overexpression of IL-10 in MF may be of pathophysiological importance for tumor progression since this immunosuppressive cytokine might be involved in downregulation of immunological tumor surveillance.

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DERMAL INTERLEUKIN 4 AND INTERFERON γ IN PATIENTS WITH ERYTHRODERMA. AN IMMUNO-HISTOCHEMICAL STUDY. Vigfús Sigurdsson, Johan Toonstra, Ilse C. Bihari, Carla A.F.M. Bruijnzeel-Koomen, Willem A. van Vloten, Theo Thepen, Department of Dermato-Allergology University Hospital, Utrecht, The Netherlands.

Erythroderma, or generalized erythema of the skin may result from different causes. At present it's unclear whether the patho-mechanisms that lead to these different types of erythroderma are identical or different. The aim of this study was to investigate the dermal T helper cell phenotypes in erythroderma.

Snap-frozen skin biopsy specimens from 34 patients with erythroderma were studied. Fourteen with idiopathic erythroderma, 7 with erythrodermic atopic dermatitis, 5 Sézary syndrome and 8 with erythroderma from miscellaneous causes. The biopsies were immuno-histochemically stained for IL-4, IFN-γ, CD4 and CD8. All positive cells in the dermis were counted and the number of positive cells was calculated per mm^2.

The results showed that the cells were mainly CD4 positive. The median CD4/CD8 ratio was 2.4 and there was no statistical difference between the groups. All groups showed more IFN-γ than IL-4 producing cells (median IL-4/IFN-γ ratio 0.6), except for the Sézary patients in which IL-4 predominated over IFN-γ (median IL-4/IFN-γ ratio 1.8). This difference was statistically significant ($p<0.05$).

Based on our results we conclude that the cells of the dermal infiltrate in patients with Sézary syndrome mainly produce IL-4 ($T_{HELPER\ 2}$ phenotype) in contrast to IFN-γ ($T_{HELPER\ 1}$ phenotype) in other types of erythroderma. This indicates that different patho-mechanisms are involved, leading to the same clinical picture of erythroderma.

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IDIOPATHIC ERYTHRODERMA: A CLINICAL, HISTOPATHOLOGIC AND FOLLOW-UP STUDY OF 28 PATIENTS. Willem A. van Vloten, Johan Toonstra, Vigfús Sigurdsson, Department of Dermatology, University Hospital, Utrecht, The Netherlands

Erythroderma may result from different causes, but a proportion remains undetermined (idiopathic erythroderma). Patients with idiopathic erythroderma have often been regarded to have a pre-Sézary syndrome because some of these patients have developed a cutaneous T-cel lymphoma (CTCL) during follow-up. The aim of this study was to investigate if this was true for our group and also if it is possible to identify further, which patients are at high risk of developing CTCL.

We analyzed clinical and follow-up data and reviewed the skin histopathology of all patients who were diagnosed with idiopathic erythroderma in our clinic between 1977 and 1994.

Twenty-eight patients, 16 males and 12 females, were diagnosed with idiopathic erythroderma. This is 27% of the patients who were diagnosed with erythroderma in our clinic, during this period. The initial histopathological examination showed two histopathological patterns: spongiotic dermatitis in 81% of the skin specimens and non-specific dermatitis in 19%. No atypical lymphoid cells were seen. During median follow-up of 33 months, 35% of the patients went into complete remission and 52% showed partial remission. Three patients (13%), all females, had persistent chronic erythroderma. Two of the latter group progressed into cutaneous T-cell lymphoma; one Sézary syndrome and one mycosis fungoides.

Based on our results we conclude that only patients with persistent chronic idiopathic erythroderma are at high risk of developing cutaneous T-cell lymphoma and therefore need a close and long-term follow-up.

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ADHESION OF MAST CELLS TO EXTRACELLULAR MATRIX PROTEINS AS COSTIMULATORY SIGNAL FOR CYTOKINE PRODUCTION. Sabine Krüger-Krasagakes, Andreas Grützkau, Silke Hoffmann, Beate M. Henz, Department of Dermatology, Virchow Klinikum, Humboldt University of Berlin, Germany.

In previous studies we have shown that immature human mast cells (HMC-1 cell line) spontaneously adhere to several matrix proteins. Moreover, mast cells are able to release a variety of cytokines upon stimulation. Therefore it was of interest to study whether adhesion processes affect synthesis and release of the proinflammatory cytokines IL-6, IL-8, TNF-α and GM-CSF. HMC-1 cells were added to fibronectin (FN)-, laminin (LN)-, vitronectin (VN)- or, for control, BSA-coated wells in the presence or absence of PMA and/or calcium ionophore A23187. For studies of gene expression, adhesion assays were stopped after 4h for isolation of RNA and semiquantitative RT-PCR analysis. Cytokine release was measured in supernatants after 24h stimulation by means of ELISAs or bioassays. Only co-stimulation of mast cells appeared to be effective, whereas incubation of mast cells on matrix proteins alone was not sufficient for the induction of cytokine gene expression or release. On BSA-coated plates, maximal cytokine expression was achieved following stimulation with PMA plus A23187. Compared to this, cytokine production was found two- to threefold increased in the presence of matrix proteins on the mRNA and also on the protein level. Only TNF-α release was not increased in culture supernatants of HMC-1 cells co-stimulated with PMA/A23187 and matrix proteins. This is possibly due to the binding of TNF-α to the matrix proteins since TNF-α mRNA levels were found increased. In summary, our results show that adhesion to matrix proteins does not initiate transcription of proinflammatory cytokine genes, but enhances gene expression and cytokine production following mast cell stimulation with an appropriate primary signal.

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EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR BY HUMAN MAST CELLS. W. Weninger, J. Pammer°, M. Baghestanian*, M. Mildner, A. Uthman, C. Ballaun, H.-C. Bankl*, P. Valent*, E. Tschachler, DIAID, Dept. of Dermatology; °Inst. of Clinical Pathology; *Dept. of Internal Medicine; Univ. of Vienna, Medical School, Vienna, Austria

Mast cells (MC) are a rich source of soluble mediators (e.g. histamine, heparin, TNF, bFGF) potentially acting on endothelial cells (EC) during tissue inflammation and neoangiogenesis. Vascular endothelial growth factor (VEGF)/vascular permeability factor has been shown to increase vascular permeability and to act as a specific mitogen for EC. When analysing the human mast cell line HMC-1 we found that these cells express VEGF mRNA constitutively. Immunoprecipitation under reducing conditions from the supernatants of HMC-1 cells revealed 3 species of VEGF protein of 15, 20, and 24 kD. To study the in situ expression of VEGF protein we used two different monoclonal antibodies. By immunohistochemistry, MC in both the skin and the intestinal submucosa were found to express VEGF protein, whereas MC in the lungs and the intestinal mucosa were negative. In situ hybridization and visualization of MC by consecutive immunofluorescence staining for tryptase on the same section revealed that approximately 15-20 % of MC in the skin expressed VEGF mRNA. In summary, our results demonstrate that human MC produce VEGF. This growth factor may play an important role in MC-mediated tissue reactions.

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INFLUENCE OF GANGLIOSIDES ON DIFFERENTIATION OF HUMAN MAST CELLS

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Gangliosides are physiological components of all vertebrate cells, mainly expressed in the outer cell membrane. The pattern is cell-type specific but compositional changes occur during physiological and neoplastic cell growth. In this study the role of gangliosides in mast cell differentiation was investigated.

Ganglioside expression in differentiating mast cell precursors from from the monocytic peripheral blood fraction as well as in HMC-1 and KU-812 cells was evaluated by extraction from the total lipid fraction in multiple steps using organic and anorganic solvents and subsequent quantitative HPTLC. In addition the influence of exogenous ganglioside GM3 on the differentiation of these cells was investigated.

After 15 days of culture in 60% horse serum (HS) and murine fibroblast supernatant (LCS), cells from the monocytic peripheral blood fraction showed a positive immunohistological staining for the high affinity IgE receptor and tryptase. GM3 was the only ganglioside expressed by these cells but in differentiated cells a 20 fold increase of the expression of the ganglioside GM3 was observed (1.2. $\mu g/10^7$ cells vs. 0.06 $\mu g/10^7$ cells, mean of 5 experiments). A higher expression of GM3 was also seen in IgE receptor positive HMC-1 and KU-812 cells compared to the IgE receptor negative cells of the same cell culture. In order to evaluate the functional role of this increase in GM3 expression, exogenous GM3 (100µg/ml) was added to peripheral blood precursors differentiating in serum depleted medium (15% HS+LCS). In comparision to controls, this led to an increase in GM3 content by $0.7 \mu g/10^7$ cells and a significant 2.5 fold increase in tryptase activity.

In summary these results point at a functional involvement of GM3 expression in mast cell differentiation.

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C3A AND C5A STIMULATE CHEMOTAXIS OF HUMAN MAST CELLS. K Hartmann, BM Henz, S Krüger-Krasagakes, J Köhl, R Burger, I Haase, U Lippert, T Zuberbier, Department of Dermatology, Virchow Clinics, Humboldt University, Berlin, Germany.

The factors that control migration of mast cells to sites of inflammation and tissue repair remain largely undefined. Whereas several recent studies have described chemotactic factors that induce migration of murine mast cells, only stem cell factor (SCF) is known to induce migration of human mast cells. Here we report that the anaphylatoxins C3a and C5a are chemotactic factors for the human mast cell line HMC-1, human cord blood-derived (CBMC) and cutaneous mast cells in vitro.

The presence of an extracellular matrix protein, laminin, was required for chemotaxis in response to complement peptides. Migration of mast cells towards C3a and C5a was dose-dependent, peaking at 1 µg/ml, and was inhibited by anti-complement antibodies. Both C3a and C5a also induced a rapid and transient mobilization of intracellular free calcium ([Ca2+]i) in HMC-1 cells. In addition to C3a and C5a, the desarginylated complement fragment C5a-des Arg also promoted chemotaxis and [Ca2+]i elevation. Other chemotactic factors tested, such as RANTES, MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β, IL-3, NGF, and TGFβ failed to stimulate migration of human mast cells.

In summary, these findings indicate that C3a and C5a serve as chemotaxins for human mast cells. Anaphylatoxin-mediated recruitment of mast cells might play an important role in hypersensitivity and inflammatory processes.

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LOCAL INJECTION OF STEM CELL FACTOR INDUCES ENHANCED MAST CELL SURVIVAL AND MAST CELL HYPERTROPHY IN TRANSPLANTED NEUROFIBROMA IN NUDE MICE. Toshio Demitsu, Tomoharu Kiyosawa, Satoru Murata and Hideo Yagita Department of Dermatology, Jichi Medical School, Tochigi-ken, Japan

It is well known that stem cell factor (SCF) plays an important role in the mast cell proliferation and development. However, little has been known the role of SCF on fully matured human skin mast cell (HSMC) in vitro and in vivo. We studied the effects of exogenous SCF on HSMCs in transplanted cutaneous neurofibroma in nude mice. Cutaneous neurofibroma tissues in a patient with von Recklinghausen's disease were sliced to 2-3 mm in diameter and transplanted into the subcutis of nude mice. Recombinant human SCF (rhSCF) (10 ng/0.1ml) was injected around the neurofibroma tissues. Mast cells in transplanted neurofibroma injected with vehicle showed decreased in number when compared to those in neurofibroma before transplantation. Local injection of rhSCF caused significantly increased mast cell number in neurofibroma tissue when compared to that injected with vehicle alone. Mast cell count in the tissue after rhSCF injection was almost as same number as that before transplantation. Thus, rhSCF recovered mast cell number up to the pre-transplantation level. One possible explanation for this phenomenon might be that rhSCF directly promotes mast cell proliferation or inhibits mast cell death rather than the induction of migration of immature or mature HSMCs into the tissue. In addition mast cell size in the neurofibroma tissue injected with rhSCF appeared significantly larger than that in both tissues injected with vehicle alone and before transplantation using the computerized image analysis.

From these findings matured HSMC is still capable of responding to rhSCF in vivo and SCF may play some role in increased mast cell number of cutaneous neurofibroma.

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SYNTHESIS AND SURFACE RECEPTOR EXPRESSION OF CD52 IN HUMAN EOSINOPHILS: A NEW DIFFERENTIATING ANTIGEN TO DISTINGUISH EOSINOPHILS FROM NEUTROPHILS. Renate Höchstetter, Karsten Spiekermann #, Alexander Kapp, and Jörn Elsner; Department of Dermatology, Hannover Medical School, Hannover, Germany; # Department of Hematology/Oncology, George-August University, Göttingen

Eosinophilic and neutrophilic granulocytes represent major effector cells in the inflammatory response. Whereas neutrophils are predominantly involved in bacterial infections, eosinophils are essential in the allergic inflammatory response. Surface markers have been used to distinguish neutrophils (CD16-positive) from eosinophils (CD16-negative) and might indicate different functional properties of these cells. In this study, expression of CD52 on human eosinophils and neutrophils was investigated in non-atopic healthy donors. Flow cytometric analysis using different anti-CD52 mAbs (mouse IgG3; human IgG1; rat IgM) demonstrated significant and homogeneous expression of CD52 on human eosinophils, but not on neutrophils. To investigate whether CD52 is expressed on mRNA level in human eosinophils, RT-PCR and Northern blot analysis were carried out. CD52 mRNA was constitutively expressed in eosinophils but not in neutrophils. Furthermore, expression of CD52 could be diminished in a dose-dependent manner by preincubation of eosinophils with phosphatidylinositol-specific phospholipase C suggesting that CD52 on eosinophils is anchored to the membrane through a glycosylphosphatidylinositol (GPI) molecule. In addition, eosinophils from 2 patients with paroxysmal nocturnal hemoglobinuria (PNH), a disorder characterized by a lack or defect of GPI-anchored membrane proteins, showed heterogeneous expression of CD52. Therefore, this study demonstrates that the GPI-anchored antigen CD52 is an efficient antigen to distinguish eosinophils from neutrophils.

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INDUCTION OF BLOOD AND TISSUE EOSINOPHILIA IN MURINE CONTACT SENSITIVITY.

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Eosinophils have been identified as important effector cells in several allergic reactions. In this study, we attempted to establish a simple mouse model of eosinophilia by epicutaneous application of a chemical hapten to investigate the mechanism of preferential production and accumulation of eosinophils.

BALB/c mice were injected sbcutaneously with cyclophosphamide (CY) (day-2) and sensitized with 7% picryl chloride (PCl)(days 0 and 1). Pretreatment of BALB/c mice with CY had the capacity to allow a strong eosinophilic response to immunization with 7% PCl. The number of blood eosinophils peaked 13 days after sensitization and was preceded by a spleen and bone marrow eosinophilia. Challenge to each ear lobe with 1% PCl on day 13 induced marked eosinophil infiltration into the dermis. This was not accompanied by an increase in the number of neutrophils. Neither blood nor tissue eosinophilia was induced by the application of 1% croton oil. VCAM-1 was transiently expressed by endothelial cells after PCl-challenge. The administration of anti-VCAM-1 Ab, but not ICAM-1 Ab abrogated eosinophil accumulation in the skin.

This mouse model of eosinophilia in response to CY followed by the sensitization with the chemical hapten should be useful in the analysis of mechanism that regulates blood and tissue eosinophilia in allergic skin diseases.

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LIPID SECOND MESSENGERS INHIBITED EOSINOPHIL OXIDATIVE BURST.

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Sphingolipids like ceramides are located mainly in plasma membranes and related organelles that are functionally associated with cellular responses to external agents like cytokines. Since decreased epidermal ceramide synthesis was demonstrated in atopic dermatitis, impaired ceramide content may also be present in the plasma membranes of effector cells. There is now much evidence that sphingolipids serve as second messengers in apoptosis and differentiation. However, little is known about sphingolipid mediated cellular effects. Ceramides were shown to modulate neutrophil effector functions, since cell-permeable C_2- > C_6-ceramide inhibited FMLP and PMA induced neutrophil superoxide formation. To investigate potential ceramide mediated effects on eosinophils representing potent effector cells in atopic dermatitis, we studied the effect of severals sphingolipids on stimulated superoxide anion production of eosinophils by *highly sensitive lucigenin-dependent chemiluminescence. It was shown that sphingomyelinase* > C_2-ceramide > D-sphingosine > C_6-ceramide = C_8-ceramide inhibited C5a induced O_2^- release in a time- and dose-dependent manner. Similar inhibitory effects were obtained when O_2^- release was induced by FMLP, PMA, PAF, IL-5, GM-CSF or TNF-α. Our results clearly indicate participation of sphingolipids in eosinophil signaling events. It is tempting to speculate that enhanced basal and stimulated superoxide generation known to be characteristic for atopic dermatitis eosinophils compared with *healthy controls may be due to impaired ceramide content. Investigations addressing* potential additional effects of lipid second messengers on other important eosinophil functions like calcium influx and apoptosis are still in progress.

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CYCLOSPORIN A INHIBITS INDUCIBLE NITRIC-OXIDE SYNTHASE INDUCTION AND TNF-a RELEASE IN HUMAN KERATINOCYTES.

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Cyclosporin A is used in the treatment of systemic and cutaneous autoimmune diseases, such as psoriasis, severe erosive lichen planus or acute GVHD, both in topical or systemic administration. Cy A acts through inhibition of IL-2 gene transcription and subsequently through inhibition of T lymphocyte activation. As iNOS is thought to be an important second messenger in the inflammatory response of various cell types including human keratinocytes, as it has been suggested that iNOS was expressed in psoriatic keratinocytes, and as we have previously demonstrated that *retinoids exerted their anti-inflammatory effect through inhibition of iNOS* mRNA expression in LPS/IFN-γ preactivated keratinocytes, we investigated the effects of CyA in LPS (1 μM)/IFN-γ (1000U/ml) prestimulated keratinocytes. The release of nitrites in culture supernatants was dose dependent (CyA: 10^{-5}-10^{-1} M) inhibited by 80% (12 μM to 3μM) after 48 and 72 h. The synthesis of TNF-α was also inhibited by 75% (140 to 30 pg/ml) after preincubation with CyA. The enzymatic conversion of ^{14}C-L-Arginine to ^{14}C-L-Citrulline was strongly down-modulated (110 pmol/mn/mg to 35 pmol/mn/mg), and this inhibition was not due to a direct effect of CyA on the enzyme activity. Actually, RT-PCR experiments confirmed that the iNOS mRNA was down modulated after an incubation of 18h with CyA. These results suggest that the clinical anti-inflammatory effects of CyA might in *part be explained by the inhibition of iNOS* gene transcription in inflammatory keratinocytes and subsequent TNF-α synthesis.

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EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) IN HUMAN EPIDERMIS OF LUPUS ERYTHEMATOSUS A. Kuhn, K. Fehsel, P. Lehmann*, T. Ruzicka* and V. Kolb-Bachofen Research Group Immunobiology in the Biomedical Center, and *Department of Dermatology, Medical Faculty, Heinrich-Heine-University Düsseldorf, FRG

The inducible nitric oxide synthase (iNOS) is known to play a key role in inflammatory and autoimmune tissue injury and in a previous study we have demonstrated expression of iNOS by epidermal keratinocytes in psoriatic skin lesions. Now we analyzed iNOS expression in skin biopsies of patients with genuine and UV-induced lesions of lupus erythematosus (LE) by immunohistochemistry and in situ hybridization. Genuine lesions of LE patients (n=20) showed in 30% iNOS mRNA and protein expression in the basal epidermal layer. Immunohistochemical staining and in situ hybridization revealed iNOS expression in 40% of UV-A-induced (n=10) and in all UV-B-induced (n=10) skin lesions of LE patients.

To address whether iNOS is expressed following exposure of skin to UV-irradiation and to define whether this photo-induced response could be involved in the formation of sunburn erythema we also analyzed skin biopsies of healthy controls (n=8) after single doses of 100 joule/cm²UV-A or 1.5 MED-UV-B. Immunohistochemistry and in situ hybridization revealed iNOS expression for up to 48 hours after UV-irradiation in the basal epidermal layer. 72 hours after irradiation iNOS mRNA and protein could no longer be detected. No iNOS expression was found in skin biopsies of untreated healthy controls (n=10).

These results demonstrate for the first time expression of iNOS by epidermal keratinocytes in genuine and UV-induced lesions of LE patients, suggesting that iNOS expression could be involved in the pathogenesis of LE. Our studies also indicate that human keratinocytes are capable of expressing iNOS for prolonged periods of time following exposure to UV-irradiation, presumably via an indirect pathway. This appears to be a major part of the integrated skin response leading to vasodilatation and erythema formation and explains the long-lasting increase in local blood-flow.

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ANTHRALIN-INDUCED ERYTHEMA IS MEDIATED BY NITRIC OXIDE. R. Parslew, L.E. Rhodes, P.S. Friedmann. Dermatology Dept., Liverpool University, Liverpool, U.K.

The mechanisms underlying anthralin-induced erythema are not fully understood. We have examined the effect of indomethacin gel, clobetasol propionate, the nitric oxide (NO) synthase inhibitor L-NAME, and fish oil rich in ω-3 polyunsaturated fatty acids (ω3-PUFAs) (a free radical scavenger) on anthralin erythema. Doubling dilutions from 0.007% to 1% of anthralin in Lassar's paste were applied to the back for 3 hours. Minimal erythema concentrations (MEC) were assessed by eye and erythema measured with a laser doppler flow meter at 24 and 48 hours. Indomethacin gel (2%) was applied under occlusion for 24 hours immediately after the anthralin was removed in 3 subjects but had no effect, indicating the lack of involvement of prostaglandins. Clobetasol propionate applied similarly caused significant inhibition of the laser doppler flux at all anthralin concentrations e.g. at 0.06% the reduction was 70% (SEM 7.1, p=0.004). L-NAME in doubling concentrations from 0.04 to 2 mM were injected into anthralin challenge sites (mid-point of the dose response curve) producing dose-related inhibition of erythema. 2mM L-NAME produced 100% inhibition compared to baseline flux levels (p= 0.009). This inhibition was obtained at both 24 and 48 hours after anthralin challenge. Since anthralin may act via a free radical mechanism, dose response curves were obtained in 10 normal subjects before and after 3 months dietary supplementation with ω3-PUFAs (known to quench free radical formation and inhibit UV-induced lipid peroxidation). *There was an increase in the MEC by one or two concentrations in 9/10 subjects* and a right shift in the curve after 3 months: eg at 0.125% p=0.04. In conclusion anthralin-induced erythema is suppressed directly with nitric oxide synthase inhibitors, topical steroids (indirectly suppressing NO production), but is unaffected by indomethacin. This suggests that NO is wholly responsible for the erythema of anthralin. The suppression by Maxepa suggests free radicals may play an important initiating role in this process.

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ANALYSIS OF THE ANTIOXIDANT ACTIVITY OF THERMAL SPRING WATER

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The purpose of this study is to investigate the biological antioxidant actions of Cauterets Thermal Spring Water, very rich in sulphurous compounds.

Antioxidant activity of Cauterets Thermal Spring Water is demonstrated by two methods. The first test uses an in vitro system : the electron spin resonance, spectroscopy coupled to spin trapping technique. The second method is a biological assay, where reactive oxygene intermediate production by macrophage is assessed by luminol dependent chemiluminescence.

These two types of investigation allow Cauterets Thermal Spring Water very strong radical scavenger properties with a dependent dose-action. This capacity is about 5 mg%g on dry matter to reduce by half the radicals concentration.

Cauterets Thermal Spring Water is effective against free radicals, which are *implicated in skin damages, in particular inflammation.*

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LEG ULCERS IN KLINEFELTER'S SYNDROME - FURTHER EVIDENCE FOR AN INVOLVEMENT OF PLASMINOGEN ACTIVATOR INHIBITOR-1. T.M. Zollner, M. Podda, M. Wolter, W.-H. Boehncke, R. Kaufmann. Dept.s of Dermatology, Univ. of Frankfurt Medical School, and Univ. of Ulm Medical School, Germany.

Abnormalities in platelet aggregability or fibrinolysis, namely elevated activity of plasminogen activator inhibitor-1 (PAI-1), have been recently documented in patients suffering from Klinefelter's syndrome associated with leg ulceration without underlying venous insufficiency. To determine whether increased PAI-1 activity is a general feature of Klinefelter's syndrome or more specifically associated with leg ulceration we investigated PAI-1 influencing parameters and PAI-1 activity in two groups of patients: (i)Klinefelter patients suffering from leg ulceration (n=7) and (ii) Klinefelter patients without leg ulceration (n=6). Analysing PAI-1 influencing parameters such as age, body mass index, triglycerids, CRP, fibrinogen, testosterone, smoking behaviour, presence of diabetes mellitus, and arterial hypertension, respectively, we found no statistically significant differences between both groups. However, *PAI-1 activity in group 1 was highly significantly elevated compared with* group two patients (p<0.005). We conclude that (i) PAI-1 activity is not elevated in Klinefelter's syndrome in general. (ii) Elevation of PAI-1 activity in patients suffering from Klinefelter's syndrome is not due to PAI-1 influencing parameters. (iii) Elevation of PAI-1 activity may play a crucial role in the pathogenesis of leg ulceration in Klinefelter's syndrome. Therefore, a therapy for leg ulceration in Klinefelter's syndrome which aims to normalize elevated PAI-1 activity should be explored.

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HHV8: A LYMPHOTROPIC, ENDOTELIOTROPIC AND NEUROTROPIC VIRUS.

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Human herpesvirus-8 (HHV-8) is the latest discovered member of the family *Herpesvirinæ*. A viremic phase preceding clinically overt Kaposi's sarcoma (KS) has been demonstrated in AIDS patients. High HHV-8 DNA loads were specifically found in primary effusion lymphomas (PELs) and in Castleman's disease.

We and others showed that secondary lymphoid organs, bone marrow, the prostate gland, KS-free skin and paravertebral sensitive ganglia appear to be privileged sites of HHV-8 latency/persistence in the infected host. Furthermore, we have shown by PCR that HHV-8 cell-associated viremia correlates with KS stage and disease activity in patients with Mediterranean KS. *In situ* studies on AIDS-KS lesions also revealed that HHV-8 selectively infects KS cells. We also obtained by *in situ* hybridization *in vivo* evidence of HHV-8 infection of neoplastic cells in 2 cases of AIDS-PELs. PCR screening of 40 AIDS-related and unrelated lymphomas confirmed very low HHV-8 detection rates. When viral load was estimated by PCR and semi-quantitative analysis, 1 case only with several cutaneous nodules and submandibular lymphnode demonstrated infection levels consistent with a PEL. This finding demonstrates that *de novo* presentation as PEL is not an universal feature of HHV-8 associated lymphomas.

Semi-quantitative PCR evaluation of HHV-8 burden in a series of HIV-negative patients with Castleman's disease revealed high loads in the plasmacellular/systemic variant. These data support the notion that a virological distinction can be made between the two forms of Castleman's disease.

Nevertheless, consistent data on the actual presence of HHV-8 in the general population are yet to come and represent an hotly debated issue.

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HHV8 IS ABSENT FROM NON-KS MUCOCUTANEOUS LESIONS OF HIV-POSITIVE PATIENTS WITH MANIFEST IMMUNODEFICIENCY

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HHV8-DNA has been detected in different forms of KS, and it has been suggested that it plays an etiologic role in the pathogenesis of this tumor. To address the question about the distribution of HHV8, we investigated the presence of HHV8-sequences in non-KS mucocutaneous lesions of HIV-infected and non-infected patients. To exclude contamination we performed nested PCR with two sets of primers in non-overlapping regions. In our study all of 28 KS specimens were positive for HHV8 by conventional PCR whereas 81 non-KS samples derived from 28 HIV-infected and 53 non-infected patients, were all negative even by nested PCR. Fourteen of the non-KS skin biopsies were derived from HIV-seropositive patients with manifest immunodeficiency i.e. with CD4 + T-cell counts of less than 50/µl. Two of these patients had simultaneous KS lesions distant from biopsy sites at the time of sampling. Conventional PCR analysis of the KS and non-KS lesions for the presence of EBV-DNA only was positive in 2 biopsies of oral leukoplakia.

Our data confirm that, HHV8 is regularily present in AIDS-associated and non-associated KS. In contrast to what has been observed in body cavity based lymphomas, we could not detect co-infection with both HHV8 and EBV in KS. Furthermore we could demonstrate that HHV8-DNA is absent from non-KS inflammatory and neoplastic mucocutaneous lesions of HIV-negative and positive patients even if overt immunodeficiency is present. Our results strongly suggest that HHV8 is highly associated with the occurence of KS and is far from being a widespread virus commonly reactivated in immunsuppressed patients.

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HUMAN HERPESVIRUS 8 DNA SEQUENCES IN SKIN TUMOURS

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Recent studies suggest the role of a new human herpesvirus (HHV8) in the pathogenesis of different forms of Kaposi's sarcoma (KS) and in both benign and malignant lympho-proliferative disorders. Since the data about the involvement of HHV8 in skin tumours other than KS are contradictory, we investigated the presence of HHV8 sequences in a large series of patients with different skin tumour tissues. DNA was obtained by proteinase K digestion from sections of formalin-fixed, paraffin-embedded tissue specimens from 84 skin lesions (basalioma, squamous cells carcinoma, actinic keratosis, angiosarcoma, haemangioma, papillary endothelial hyperplasia, angiokeratoma, pyogenic granuloma, spindle cell haemangioendothelioma, angiolymphoid hyperplasia with eosinophilia, lymphangioma and haemangiopericytoma). The DNA was then amplified by using primers specific for HHV-8 DNA. The polymerase chain reaction (PCR) products were analysed on 1.5% agarose /ethidium-bromide. The specificity of the bands were confirmed by hybridization to a previously sequenced HHV8 probe. In positive cases the PCR products were cloned and direct sequenced. HHV8 DNA sequences were present in two tumours of patients with angiosarcoma of the face confirming our previous case report (Gyulai et al. N Engl J Med, 1996). In addition, HHV8 sequences were found in a rare benign tumour of atypical endothelial cells in each of the four tested patients with angiolymphoid hyperplasia with eosinophilia (ALHE). HHV8 sequences could not be detected from the other skin tumours. The detection of HHV8 in both benign ALHE and malignant (KS and angiosarcoma of the face) lesions suggest the virus alone is not sufficient to produce a specific lesion; it might rather be the trigger of a complex reactions.The role of HHV8 in inducing proliferative diseases demand further elucidation.

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INFECTION OF KERATINOCYTES IN VITRO WITH EPSTEIN-BARR VIRUS

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The Epstein-Barr virus (EBV) is a herpes virus which can infect not only B and T lymphocytes but egaly epithelial cells. Recently, EBV sequences have been identified in keratinocytes with an in situ hybridization technique (ISH) in skin biopsies.

The aim of our study was to confirm the capacity for EBV to infect human normal keratinocytes cultived in monolayer.

In a first step, we studied EBV fixation on keratinocytes in vitro. For this, EBV was obtained from B 95-8 cell, purified and labbeled with biotin (b-EBV). Then, b-EBV was incubated 30 minute with keratinocytes and revealed with streptavidin-FITC. We found a granular membran fluorescence on 30% of keratinocytes, whereas none melanocyte cells used as negative control was positive. In a second step , monolayer keratinocyte cultures were incubed 2 hours at 37°C with EBV, then the cultures were washed and continued 7 days with fresh free EBV medium. EBV infection of keratinocyte was looked for both by in situ hybridization with EBER/BHLF probes, immuno-histochemistry (expression of latent antigen LMP) and PCR . EBER was identified in 10% keratinocytes, LMP was expressed in 40% cells and PCR was positive (negative in non-incubated keratinocytes used as control).

In conclusion, this study demonstrates a fixation of EBV on keratinocytes raising the question of the nature of the receptor on keratinocyte (CD21?), and the capacity of EBV to infecte keratinocytes in vitro. In vitro keratinocytes appear of interest as model for studying activation of epithelial cells by a viral infection.

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UVB-IRRADIATION PROVOKES TRANSCRIPTIONAL ACTIVATION OF ENDOGENOUS RETROVIRAL SEQUENCES IN HUMAN KERATINOCYTES

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The human genome contains at least 1% endogenous retroviruses and retroviral elements (HERVs) sharing structure and sequence homologies with exogenous retroviruses. Human chromosomal DNA includes, besides HERVs in the proviral form, also a large numer of solitary LTRs harboring all the control elements necessary for gene expression. Therefore, HERVs represent a reservoir of potentially pathogenic viral sequences which may be activated spontaneously or by environmental influences. The influence of UV-irradiation (UVA: 320-400 nm; UVB 280-370 nm) on expression of endogenous retroviral sequences and LTR-driven transcription of cellular genes was investigated using a human keratinocyte cell line (HaCat). Two different methods were applied: 1.) Conserved sequences contained in the retroviral *pol* gene were amplified from cellular polyA(+)-RNA performing RT-PCR with degenerated oligonucleotide primers. These PCR products were hybridized in a second step to distinct *pol* fragments of 96 different HERVs (reverse dot-blot hybridization; Herrman & Kalden, 1994, *J. Virol. Methods*, 46:333-348). Using this method, the basal expression of six HERVs, belonging mainly to the ERV9 family, was detected in untreated HaCat cells. Furthermore, it was shown that irradiation of these cells with 30 mJ/cm^2 UVB leads to transcriptional activation of a number of additional endogenous viral sequences. These sequences hybridized with at least two retroviral transcripts previously detected in autoimmune patients. In contrast, irradiation with UVA using up to 20 J/cm^2 had little or no effect on HERV expression. 2.) In order to compare HERV-related expression pattern in UV-irradiated and non-irradiated HaCat cells, a modified *differential display* technique was established. This approach will enable the isolation of HERV LTR-initiated cellular transcripts. Analysis of the expression of endogenous retroviruses in relation with environmental influences may help to elucidate their role in the pathogenesis of certain skin tumors and autoimmune diseases, e.g. lupus erythematosus and Sjögren's syndrome.

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ERADICATION OF CANINE ORAL PAPILLOMATOSIS VIA INTRA-LESIONAL NAKED IFN-A DNA: RESULTS OF A DOUBLE-BLIND TRIAL.

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Epidermal keratinocytes take-up and express naked plasmid DNA which has been injected into the dermis. Using this technique, genes encoding biological response modifiers, such as interferons, could be used to treat a variety of skin lesions. To assess the efficacy of this approach, we treated buccal mucosa papillomas caused by canine oral papilloma virus (COPV) with plasmid DNA encoding canine interferon-α (IFN-α). Initially, the indicator gene ß-galactosidase driven by a CMV promoter (pCMV:ßGal) was injected into canine buccal mucosa to assess both the time course and magnitude of expression. The ßGal protein was detected as early as three hours after injection, with maximal levels (3000ng per 8mm injected area) present at 6 hours. βGal staining was lost from the buccal mucosa within 48 hours due to its high turnover. To study the effect of injected canine IFN-α plasmid DNA on papillomas, 2-3 month old beagles were inoculated with wart extracts containing COPV. In a double-blind study, a total of 40 dogs were treated with 1x10^6 IU recombinant human IFN-α protein, 50µg canine IFN-α plasmid DNA, 50µg control vector DNA, or PBS. Treatments were administered every other day for 3 weeks or until complete remission of the lesions occurred. Complete regression has been seen in 85% of warts treated with canine IFN-α plasmid DNA (mean of 13 days), and in 90% of lesions treated with recombinant human IFN-α protein (mean of 11 days). Occasionally regression was observed in warts treated with either control vector DNA or PBS.

These results suggest that injection of plasmid DNA encoding IFN-α is a viable approach to the treatment of cutaneous disease. Clinical trials have to compare the efficiency of recombinant protein with plasmid DNA expressing human IFN-α.

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ESTABLISHMENT OF A HUMAN PAPILLOMAVIRUS IMMORTALIZED KERATINOCYTE CELL LINE FROM A SQUAMOUS CELL CARCINOMA OF A RENAL TRANSPLANT RECIPIENT.

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In renal transplant recipients (RTR) the role of specific human papillomavirus (HPV) types in the pathogenesis of squamous cell carcinoma (SCC) is now well recognised. Several known and unknown HPV types have been identified in benign and neoplastic lesions of RTR. In this study, we have established a keratinocyte cell line from an SCC from a RTR and have examined it for the presence of HPV.

A fresh SCC biopsy was taken from the face of an RTR. The tissue was decontaminated, finely minced and cultured in modified Rheinwald-Green media under coverslips. 11 cell lines were established which are independant of growth factors. Total DNA was extracted from each cell line and used as template for polymerase chain reaction (PCR) with specific HPV degenerate primers HVP2 and B5. Specific bands (600-700bp) were visualised by agarose gel electrophoresis and positive PCR products were cloned and sequenced.

To date a 98 bp sequence from one of the 7 positive cell lines has been analysed using polyacrylamide gel electrophoresis. This sequence included one of the specific primers used, and when screened against the European Molecular Biology Laboratory sequence data base, was found to share 63.2% homology with HPV 51.

These results provide further support for HPV involvement in the development of SCC in RTR. This virus may also be an important agent in immortalizing keratinocyte populations.

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DETECTION OF HPV-DNA IN TRICHILEMMOMAS BY POLYMERASE CHAIN REACTION.

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To date it is still a point of discussion whether trichilemmomas are true benign neoplasms or virus-induced hyperplasias. Therefore we investigated paraffin sections of 11 typical trichilemmomas for the presence of HPV-DNA by PCR with different degenerated consensus primer pairs (MY 11/09 and CP65/70). Amplification products of the expected size for all 11 cases investigated were found by modification of PCR conditions. The eleven amplicons were cloned and sequenced. Comparison of the deduced amino acid sequences to all known HPV-types classified these as members of the EV associated HPV's in 9 cases (especially HPV 15, 17, 23 and 37) whereas two cases contained HPV-6b. These results demonstrate for the first time the presence of HPV-DNA in trichilemmomas and support the hypothesis of trichilemmoma as a viral induced hyperplasia with a predominance of EV-associated HPV-types.

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Human Papillomavirus Infection (HPV) in Cured Hodgkin's Disease Patients

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Patients suffering from Hodgkin Disease (HD) have an increased risk of development HPV-associated lesions.We examined 50 Hodgkin's Disease (HD) patients clinically, after succesful treatment of their disease. Serum was available from 45 patients. Twenty-two patients (44%) had HPV-associated skin and mucosal lesions like warts (38%), condylomata acuminata (12%), epidermodysplasia verruciformis (4%) or erythroplasia of Queyrat (4%). 10% have possible HPV associated basal cell carcinoma or other skin disorders. Almost all premalignant and malignant lesions were located on skin areas that had been treated with radiotherapy.

In order to evaluate seroreactivity against oncogenic HPV types, we analyzed the serum of the patients for antibodies against HPV8 L1 using Western Blot (48% positive) and HPV16 E4 and E7 using Peptide-ELISA (29.5% positive) or RIPA (48% positive).

Since cured HD patients face an increased risk of secondary malignancies, the clinical demonstration, especially of precancer and cancer skin lesions and the serological demonstration of potentially oncogenic HPV types, underline the importance of careful follow-up of these patients.

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MIB-1 EXPRESSION IN BUSCHKE-LÖWENSTEIN TUMOURS AND CONDYLOMATA ACUMINATA

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Benign condylomata acuminata and locally destructive Buschke-Löwenstein tumours show similar histologic features and discrimination between these HPV-induced tumours relies on the clinical aspect and clinical course of the tumours. As proliferating cells express MIB-1 and as MIB-1 reactivity has been used to discriminate between CIN I, II and III lesions, we analysed the expression of MIB-1 in 2 HPV 6-induced Buschke-Löwenstein tumours and in 10 condylomata acuminata. We observed a bimodal distribution of MIB-1 reactivity in the tumours as MIB-1 expression could be demonstrated in the basal keratinocytes as well as in the differentiated non-dividing upper spinous cells. No difference in the MIB-1 reactivity could be found in benign condylomata acuminata as compared to the clinically malignant Buschke-Löwenstein tumours. Analysis of 2 condylomata acuminata and one Buschke-Löwenstein tumour harbouring mutated HPV 6 genomes with duplications within the upper regulatory region (URR) did not show an increase in the MIB-1 expression as compared to tumours harbouring the wild type HPV 6 DNA. Our results suggest that MIB-1 reactivity does not discriminate between proliferating cells and non-dividing cells which have activated their DNA replication machinery in order to replicate viral DNA. Furthermore, condylomata acuminata and Buschke-Löwenstein tumours do not seem to differ in their activation of the DNA replication machinery.

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THE ANTIBACTERIAL PEPTIDE LL-37 IS PRESENT IN SKIN AND IS INDUCED IN THE EPIDERMIS DURING INFLAMMATION

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Antibacterial peptides are part of the innate immune system. They are immediate actors with a broad antimicrobial spectrum. In its standby position innate immunity contributes to the first line defense against infectious agents and jumpstarts the adaptive immune response. In human, five antibacterial peptides have been characterized from granulocytes; four defensins (HNP-1-4) and LL-37. The antibacterial peptide LL-37 belongs to the cathelicidins, a protein family with a conserved proregion (cathelin) and a variable C-terminal antibacterial peptide. Skin is a major barrier to the environment and serves a putative protective role against microbial invasion. We have recently isolated antibacterial peptides and proteins in wound and skin blister fluids. In the present study, the antibacterial peptide LL-37 is detected in skin extract by immunoblotting. For cellular localization, we used immunohistochemistry, and LL-37 is found in sweat glands and in single granulocytes in normal skin, but not in the epidermis. However, in various inflammatory skin diseases, strong immunoreactivity as well as signal for LL-37 mRNA is demonstrated in the epidermis. Our results indicate that LL-37 is induced in keratinocytes during inflammation and contributes to the microbial defense system of the skin.

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THE VALUE OF NITRO-BLUE-TETRAZOLIUM TEST IN THE DIAGNOSIS OF RELAPSING PYODERMAS

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The purpose of our work was to study the modifi - cation of some parameters of the specific and non-specific immunity in 31 patients with relapsing pyodermas such as furuncles, hidradenitis. The parameters were: total leukocyte count and differential count, the nitro-blue-tetrazolium test, the amount of immunoglobulines, circulating immune complexes and the ratio T cells/B cells, T helper/T suppressor.

The nitro-blue-tetrazolium test /NBT/ alloweds to establish the amount of active metabolic leukocytes.

The most important deficiency was shown by the nitro-blue-tetrazolium test /6o % of the patients with reduced values of NBT-positive leukocytes/. There were also found patients with leukopenia /23,3 %/,neutropenia /16,6 %/,modifications of lymphocyte ratio /lo %/, deficiency of Iga /6,66 %/.

In our opinion, relapsing pyodermas are due mainly to the deficiency of non-specific immunity, especially deficiency of oxido-reductive metabolisme of leukocytes. The test NBT proved important in the diagnosis of immune modifications in patients with relapsing pyodermas.

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MORPHOLOGICAL TRANSITION IN PITYROSPORUM OVALE. T.Bhattacharyya[1], M.D.Richardson[1], M.Edward[1], C.S.Cordery[2], C.Morris[2]

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The high proclivity of hyphae in pityriasis versicolor, where filaments aid invasion of squamous epithelial cells,may indicate a pathological significance of hyphae in the pathogenesis of other *Pityrosporum ovale*-related diseases, such as seborrhoeic dermatitis and dandruff. Studies on the yeast-to-hypha (Y-M) transition in *P.ovale* have revealed a number of factors capable of producing filamentation in various isolates. An environmental "morphogen" that acts alone under all conditions to promote filamentation in *P.ovale* isolates has yet to be discovered.The increasing significance of *P.ovale* as a human pathogen has prompted the need for a reevaluation of conditions shown to induce Y-M transformation and to examine further whether invasive growth maybe potentiated by the capacity of *P.ovale* to form hyphae.

Growth in a 10% CO_2 atmosphere caused up to 8 % germination in some strains. Addition of various sebum components such as oleic acid, squalene and cholesterol to stripped stratum corneum or living skin equivalents where morphology in the presence of stratum corneum cells could be observed, caused changes in morphology and up to 20% germination of yeast cells. Other additives such as divalent cations were not found to stimulate filamentation. Filamentation media (Dom,1977), previously shown to stimulate hyphal growth in strains of *P.orbiculare* alone, stimulated an isolate from scalp psoriasis to produce 20-34% filamentation after 1 weeks incubation at 29°C. Of the filaments observed, 31% were branched and by filtering and reinoculation of the filamentous isolate in filamentation media, the percentage of filamentous cells was increased to 75%.

This study has highlighted the difficulties in stimulating Y-M transition for *P.ovale in vitro*.

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IN SITU PCR, PCR IN SITU HYBRIDIZATION: NEW DIAGNOSTIC TOOLS IN DERMATOPATHOLOGY.

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The polymerase chain reaction (PCR) serves as an important technique in a laboratory of dermatopathology. Particularly, the detection of viral DNA in formalin-fixed and paraffin-embedded material is a major application for PCR analysis. Despite these and other exciting prospects conventional PCR technique bears some disadvantages. The possibilty of contamination and the lack of assignment of a positive signal to a particular cell which contains the DNA being amplified. In situ PCR and PCR in situ hybridization are recently developed techniques which overcome these difficulties. In skin biopsies of a patient suffering from a generalized infection with the varicella zoster virus we could demonstrate the virus by conventional PCR. By in situ PCR and PCR in situ hybridization we could visualize the localization of the virus using paraffin-embedded slides. The quality of the in situ PCR assay was slighthly inferior compared to the PCR in situ hybridization, due to a stronger background. We conclude PCR in situ hybridization and in situ PCR are new important tools for demonstration of viral DNA in paraffin-embedded material on a routine basis.

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PROOPIOMELANOCORTIN PRODUCTS ENHANCE HISTAMINE AND LTC_4 RELEASE FROM THE HUMAN CELL LINE HMC 1 AND HUMAN CUTANEOUS MAST CELLS IN VITRO

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Proopiomelanocortin (POMC) is a pituitary opioid peptide precursor that undergoes enzymatic cleavage into peptides such as α-MSH, ACTH and ß-endorphin. Several resident and transient cutaneous cell populations have recently been shown to express melanocortin receptors corresponding to POMC products. Furthermore, POMC products are known to be processed and secreted in the skin in vivo and by various skin cells in vitro. Although mast cells are thougt to be involved in neurohormonal processes, these cells have so far not been studied in this regard. We have therefore stimulated the human leucemia cell line HMC-1 and cutaneous mast cells derived from dispersed human skin with ACTH, ß-Endorphin and α-MSH with and without pre-stimulation with SCF, NGF, PMA or TNF-α. All three POMC products caused a significant dose- and time-dependent histamine and LTC_4-release that was comparable to values obtained after stimulation with anti-IgE, which was used as positive control. Mediator release could be enhanced by pre-stimulation with PMA. Preliminary attempts aimed at elimidating the mechanisms involved during release failed to show intracellular Ca^{2+} release after stimulation with POMC products using fluorometrical determination. This suggests that G protein related signal transduction pathways are probably not involved. These results demonstrate thus for the first time a possible receptor mediated effect on mast cell activation by POMC products which will have to be further elimidated regarding its mechanisms and biological relevance.

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SELECTIVE STIMULATION OF METHIONINE-ENKEPHALIN SYNTHESIS IN HUMAN MONOCYTES. J.B. Nissen, K. Stengaard-Pedersen, K. Kragballe. Department of Dermatology, Marselisborg Hospital, University of Aarhus, Aarhus, Denmark

Opioid peptides are synthesised in neurons, endocrine cells and immune cells. Beside antinociception, they modulate immune reactions and cell proliferation. As previously shown the number of enkephalin-producing monocytes/macrophages are increased in psoriatic lesions compared to uninvolved skin and normal skin. Therefore, the purpose of this study was to investigate whether compounds known to stimulate monocytes are able to induce enkephalin synthesis. Monocytes ($2x10^6$ /ml, >85% CD68 positive cells), isolated from citrate-treated blood by Ficoll-Hypaque density gradient centrifugation followed by adherence to plastic, were incubated with lipopolysaccaride(0.2-10µg/ml), TNFα(0.1-10ng/ml), PMA(10^{-6}-10^{-11}M) and γIFN(1-10ng/ml) in RPMI-1640, 10% human ABserum for 18h. After centrifugation proteins were extracted from the cell pellet in 0.2 M HCl/DDT(0.005M) by homogenisation and heating for 3 minutes at 100°C. Following separation by HPLC, the amount of enkephalin was determined by radioimmunoassay. Cell viability, as determined from trypan blue exclusion, was >90%. To detect any spontaneously enkephalin synthesis, isolated monocytes were stained with antisera against enkephalin and CD14/CD68. There was no or only a faint staining for enkephalin in CD14 positive cells and this was similar for freshly isolated cells and cells incubated with medium. Stimulation of monocytes with LPS induced a strong dose-dependent production of α-enkephalin. PMA induced a weaker α-enkephalin production, while TNFα and γIFN had no effect. Our results show that human monocytes can be stimulated to produce enkephalin in vitro, and that this stimulation is selective for certain monocyte stimuli. The increase of enkephalin producing monocytes/macrophages in psoriatic skin lesions may, therefore, result from the presence of selective stimuli not present in uninvolved skin.

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ACTIVATION OF THE HUMAN MC1 RECEPTOR BY SKIN ACTH PEPTIDES. K Wakamatsu, A Graham, G Hunt, S Kyne, D Cook*, AJ Thody. Departments of Dermatology & *Clinical Biochemistry, University of Newcastle upon Tyne, UK.

Pituitary derived pro-opiomelanocortin (POMC) peptides are produced at various extra-pituitary sites including the skin. Of these peptides α-melanocyte stimulating hormone (α-MSH) is well known for its melanogenic action which is mediated by the G-protein coupled MC1 receptor (MC1-R) present on melanocytes. Another POMC peptide, adrenocorticotrophin (ACTH1-39) also stimulates melanogenesis in human melanocytes. The present study was carried out to see whether ACTH peptides are capable of activating the human MC1-R and whether they are present in the skin. The ability of ACTH peptides to activate the MC1-R was examined in cultured human melanocytes and HEK293 cells that had been stably transfected with the human MC1-R. Of the peptides tested ACTH1-39 and ACTH 1-17 were as active as α-MSH in stimulating melanocyte dendricity and melanogenesis. These peptides also increased intracellular levels of cAMP with the following order of potency: ACTH1-17 > α-MSH > desacetyl α-MSH > ACTH1-39 > ACTH1-10. Using immunocytochemistry and an α-MSH specific antibody we confirmed the presence of this peptide in human epidermis. Immunostaining was evident in most keratinocytes and especially those in the upper layers of the epidermis. Strong staining was noticeable in isolated dendritic cells in the basal and suprabasal layers and occasionally in cells within the dermis. A similar pattern of staining was seen following immunostaining with antibodies directed to ACTH1-10, ACTH1-39, and ACTH17-39 demonstrating the presence of ACTH peptides in human epidermis. HPLC coupled with radioimmunoassay confirmed the presence of α-MSH, ACTH1-39 and ACTH1-10 in cultured keratinocytes. The present findings demonstrate that ACTH peptides as well as α-MSH are present in human epidermis and that these peptides are capable of activating the human MC1-R. The precise functions of ACTH peptides in the skin are not yet clear but it seems likely that together with α-MSH, they act to regulate melanocytes.

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THE HAIR FOLLICLE AS A SOURCE AND TARGET OF NEUROTROPHIC FACTORS

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Neurotrophins (NT) not only control the development of peripheral tissue innervation, but may also be involved in the regulation of epithelial tissue development and growth. The epithelium, in turn, is an important source of NT. Using the murine hair follicle as a model for studying the role of epithelia as sources and targets of NT, we have analyzed the antigen expression of the following NT and NT receptors during the induced hair cycle of C57BL/6 mice: NGF, NT-3, NT-4, brain derived neurotrophic factor (BDNF), TrkA, TrkB, TrkC, p75. We found that, in telogen skin, NGF-, NT-3-, and TrkA-immunoreactivity (IR) is expressed in the hair bulge cells. In addition, NGF- and NT-3-IR was detected in Schwann cell-like processes located around follicular isthmus, while BDNF- and TrkC-IR observed only in subcutaneous nerve bundles during telogen. During early anagen, NT-3-IR keratinocytes appeared in the outer root sheath (ORS) of the hair bulb, accompanied by NT-4- and p75-IR in the perifollicular Schwann cell processes and the presence BDNF-IR keratinocytes in the epidermal basal layer and in the follicular infundibulum. During anagen VI, keratinocytes of the ORS and hair bulb were NT-3- and NT-4-IR, while the dermal papilla of anagen VI follicles was TrkB- and TrkC-positive, and the inner root sheath was to be BDNF-IR. During the anagen-catagen transformation, we found BDNF-, TrkA-, and TrkC-IR (early catagen), NT-3-, BDNF-, NT-4-, TrkA-, and TrkB-IR (late catagen) in regressing hair bulb keratinocytes. The arrector pili muscle expressed strong NT-3-, BDNF- and TrkB-IR during the entire hair cycle. These results suggest that the hair follicle is major source of NT, and that its secretion of neurotrophic factors and expression of NT receptors are tightly regulated. NT may be an important regulatory factor in the epithelial-mesenchymal-neuroectodermal interactions that drive the hair cycle.

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CGRP-RECEPTOR ANTAGONIST INHIBITS DELAYED HYPERSENSITIVITY REACTIONS. Joanna Wallengren, Department of Dermatology and Pharmacology, Lund University Hospital, Lund University, S-22185 Lund, Sweden.

There is growing evidence that the neuroendocrine system can influence immune functions. We have previously shown that specific antagonist to substance P, spantide, inhibits immediate and delayed hypersensitivity reactions. Substance P coexists with calcitonin gene-related peptide, CGRP, in sensory nerve fibers. Both neuropeptides are co-released from sensory nerve fibers in response to physical and chemical irritants.
We have studied the effect of CGRP and a selective CPRP receptor antagonist (CGRP 8-37) on delayed hypersensitivity reactions in individuals with nickel allergy. CGRP in the given amounts did not affect the immune responses while CGRP (8-37), given in fourfold greater amounts than CGRP, inhibited the reactions to nickel.
A possible explanation for these results is that CGRP contributes to the symptoms of contact dermatitis together with substance P, and that the contribution of CGRP can be eliminated by pretreatment with the antagonist.

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CUTANEOUS REINNERVATION AFTER DILTIAZEM THERAPY FOR VIBRATION WHITE FINGER. S Chopra, H A Bull, P C Goldsmith, * J C Foreman & P M Dowd. Academic Unit of Dermatology, Department of Medicine & * Department of Pharmacology, UCL Medical School. London. UK.

We have previously reported a depletion of Calcitonin Gene Related Peptide containing nerves in the digital cutaneous biopsies of subjects with vibration white finger (VWF). Calcium channel blockers such as Diltiazem are commonly used in the therapy of Raynaud's phenomena of differing aetiology. We have recently found that patients with VWF treated with Diltiazem for 18 months report a sustained reduction in the number of blanching attacks per week even after discontinuing Diltiazem for one week. In addition, these patients had larger and more intense histamine and Endothelin-1 induced flares post Diltiazem therapy. This has led us to hypothesise that long term Diltiazem therapy may partially reverse the cutaneous CGRP neuronal loss in VWF. We have carried out a quantitative immunohistochemical study to test this hypothesis.

Digital cutaneous biopsies were taken from the distal or proximal phalanx of affected digits of patients with VWF prior to commencement of Diltiazem. The patients used the highest tolerable doses (60-180mg/day) for the winter months. Digital cutaneous biopsies were performed 3mm distal or proximal to the pretreatment biopsies after 18 months of Diltiazem therapy and after having ceased to take Diltiazem for at least two weeks.

A standard DAB peroxidase technique was used to immunostain for PGP 9.5 (a pan-neuronal marker) and for CGRP on the pre and post treatment biopsies. Quantitative analysis showed that the post treatment biopsies had greater immunostaining for PGP 9.5 although this was not significant, however the quantitative analysis for CGRP neurons showed a significantly greater area of immunostaining in the post treatment biopsies. We believe that this may represent at least a partial reversal of the cutaneous neuronal deficit found in VWF after treatment with Diltiazem.

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EXPRESSION OF SOMATOSTATIN RECEPTORS ON HUMAN DERMAL FIBROBLASTS. A.Gaudillère[1], L.Misery[1,2], C.Bernard[3], J.Abello[3], A.Claudy[2,1], D.Schmitt[1,1] INSERM U346 ; [2] Department of Dermatology ; [3] INSERM U45 ; E.Herriot Hospital, LYON, FRANCE.

Somatostatin (SOM) is a neuropeptide that could exert its effects directly through receptors on target cells. Two classes of receptors have been identified depending on their affinity for SOM or octreotide (a SOM agonist). Dermal SOM immunoreactive cells can be identified by immunohistochemical methods. It is the reason why we have searched for SOM receptors on human dermal fibroblasts.
Foreskin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated calf serum and L-glutamine. After 4-6 passages, confluent fibroblasts were separated with trypsin/EDTA solution. Radioligand binding studies were carried on these fibroblasts and RINm5F (as positive control) using (3-(^{125}I)iodotyrosyl^{11})-SOM-14. Non-specific binding was determined with 10^{-5}M SOM-14. Scatchard analysis were performed with increasing concentration of the tracer and displacement study with increasing concentrations of SOM-14, SOM-18 and octreotide.
Binding of (3-(^{125}I)iodotyrosyl^{11})-SOM-14 to dermal fibroblasts was time and temperature dependant. Maximal specific binding was achieved at 22°C after 6 hours. Scatchard analysis of equilibrium binding curves showed a curvilinear relationship.
We conclude that the two classes of SOM receptors are present on human dermal fibroblasts and that theses receptors may explain the role of SOM or octreotide in skin diseases such as psoriasis or systemic sclerosis.

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ALPHA-MELANOCYTE STIMULATING HORMONE MODULATES MELANOCORTIN RECEPTOR-1 EXPRESSION AND UPREGULATES IL-8 PRODUCTION IN HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS (HMEC-1).
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Dermal microvascular endothelial cells are important sources of proinflammatory cyto- and chemokines and mediate leukocyte-endothelial interactions during cutaneous inflammation. Recent studies revealed a crucial role of neuropeptides such as α-melanocyte stimulating hormone (α-MSH) within the skin immune system. This peptide hormone is generated by posttranslational enzymatic cleavage of the precursor molecule proopiomelanocortin (POMC). The effects of POMC-peptides are mediated by a group of G-protein-coupled receptors known as melanocortin (MC-) receptor 1 - 5. In contrast to the other receptors, MC-1 is specific for α -MSH only. The purpose of the present study was to investigate the melanocortin receptor expression in human dermal microvascular endothelial cells (HMEC-1) by performing RT-PCR using melanocortin receptor specific primer pairs. Accordingly, unstimulated HMEC-1 cells express constitutively MC-1. Additionally, this expression could be upregulated upon stimulation with IL-1β and α-MSH itself as indicated by semiquantitative PCR-studies and northern blots. Neither in unstimulated nor in IL-1β, PMA or α-MSH stimulated cells MC-2, -3, -4 or -5 expression could be detected. In order to evaluate the physiological relevance of MC-1 expression, HMEC-1 were treated with various concentrations of α-MSH (10^{-6} to 10^{-15} M) and analysed regarding cyto- and chemokine-production using specifical ELISAs. α-MSH (10^{-8} - 10^{-10} M) after 1 hr significantly upregulated expression of IL-8-mRNA in HMEC-1 followed by an enhanced release of this chemokine after 8 - 10 hrs. Competition binding studies using ^{125}J-α-MSH and different competitor peptides verified, that the observeable effects are due to specific and saturable binding of α-MSH to HMEC-1. These data provide first evidence that microvascular endothelial cells express functional melanocortin receptors which may play a role in inflammation.

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KERATINOCYTE (HaCat CELL) DIFFERENTIATION MEDIATED Hsp70 EXPRESSION IS CONTROLLED BY ALPHA MELANOCYTE STIMULATING HORMONE.
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Heat shock proteins are versatile tools engaged in a variety of cellular functions. Particularly, the stress inducible 70 kDa heat shock protein (hsp70) not only confers protection to cells but also is involved in the regulation of the production of cellular stress response mediators such as cytokines. In addition to cytokines, neurohormones such as the proopiomelanocortin derived αmelanocyte stimulating hormone (αMSH) recently turned out to be potent mediators of inflammatory and immune responses. Thus the present study was performed to investigate the role of αMSH on the expression of hsp70 in a human keratinocyte cell line (HaCaT). The proliferation and differentiation of HaCat cells can be regulated by changing extracellular Ca^{2+} concentrations. HaCaT cells induced to differentiate in high Ca^{2+} medium (1.5mM) were found to express higher levels of hsp70 protein in comparison to cells grown under low Ca^{2+} conditions. Moreover, differentiated HaCaT cells were markedly more resistant to oxidative stress as compared to undifferetiated control cells. αMSH in a dose dependent manner significantly suppressed hsp70 expression in differentiated HaCaT cells, but only had a minor effect on undifferentiated cells. HaCaT cells grown in high Ca^{2+} medium upon treatment with αMSH were renderd more sensitive to oxidative stress, which significantly decreased their survival rate. These findings indicate that αMSH which is released by keratinocytes upon injurious stimuli such as tumor promoters or ultraviolet light in an autocrine fashion is able to regulate their cytoprotective protein equipment.

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AGOUTI PROTEIN INHIBITS EUMELANIN AND PHAEOMELANIN PRODUCTION IN B16 MOUSE MELANOMA CELLS. A Graham, K Wakamatsu, G Hunt, S Ito*, AJ Thody. Dermatology Department, University of Newcastle upon Tyne, UK and *School of Health Sciences, Fujita Health University, Toyoake, Aichi 470-11, Japan.

In mammals the relative proportions of eumelanin and phaeomelanin are regulated by α-Melanocyte stimulating hormone (α-MSH) which acts via its receptor on melanocytes to preferentially increase the synthesis of eumelanin and by the Agouti protein (AP) which antagonises this action. There is evidence that AP is also capable of inhibiting melanogenesis by an α-MSH-independent action. In this study we have examined how these two mechanisms affect the pattern of melanogenesis. α-MSH (10nM) increased tyrosinase activity and melanogenesis in cultured B16F1 mouse melanoma cells with eumelanin content increasing by 352% and that of phaeomelanin by 15%. Recombinant mouse AP (0.1-100nM) reduced these effects of α-MSH in a dose-related manner and at the highest concentration (100nM) eumelanin and phaeomelanin contents were reduced to near pre-MSH control levels. AP also blocked the increase in intracelular cAMP induced by α-MSH, but failed to reduce the melanogenic effects of dibutyryl cAMP (1mM). These findings are consistent with the view that AP acts by opposing the action of α-MSH at the receptor level. However, AP also produced dose-related decreases in tyrosinase activity and melanogenesis in the absence of α-MSH and these changes were accompanied by reductions in the contents of both eumelanin and phaeomelanin. Decreases in eumelanin and phaeomelanin in response to AP were also seen in B16G4F cells that lack MSH receptors. These findings demonstrate that AP decreases melanogenesis and the synthesis of both eumelanin and phaeomelanin by opposing the action of α-MSH and also by a second mechanism that is independent of α-MSH.

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STRUCTURAL AND MOLECULAR CHANGES IN HUMAN SKIN DURING UV-STIMULATED MELANOGENESIS. JM Naeyaert, M De Mil, J Lambert, Department of Dermatology, University Hospital, Gent, Belgium.

We have previously reported that there is no correlation between pigment content and tyrosinase message level in cultured normal and malignant human melanocytes under basal and stimulated conditions and that the rate of melanogenesis is determined by post-translational events such as activation of preexisting tyrosinase enzyme and/or other proteins[*]. However, little is known on the role of tyrosinase in regulating melanogenesis *in vivo*. We therefore irradiated 10 Caucasian volunteers (4F, 6M - aged 27-61 years - skin phototype III) with a single 4 MED UVB-dose on the buttocks. Skin was harvested at day 2, 4 and 10 post-irradiation by taking suction bullae and classical biopsies, with non-irradiated buttock skin as control. No increase in the mean number of epidermal melanocytes was seen in irradiated skin. There was a progressive increase in melanin content and tyrosinase activity. At day 10, L-DOPA-staining of epidermal sheets was very intense and showed huge, very dendritic melanocytes. Semi-quantitative RT-PCR with DNAse-treated RNA extracted from the bullae roofs revealed a relative increase in tyrosinase mRNA-level on day 4 and 10, but not on day 2 post-irradiation. These results favour the hypothesis that during a first phase a preexisting pool of tyrosinase is being activated without a change in the constitutive levels of tyrosinase mRNA. In the second phase of the response, starting at day 4 post-irradiation, steady state levels of tyrosinase mRNA-levels begin to rise as the pool of tyrosinase is being depleted.

(*) JM Naeyaert, M Eller, PR Gordon, HY Park, BA Gilchrest : Pigment content of cultured human melanocytes does not correlate with tyrosinase message level. Brit.J.Dermatol. 125 : 297-303, 1991.

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Melanocytes from vitiligo patients have alterations in the antioxidant system and are more susceptible to oxidative stress

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Recent reports have suggested that oxidative stress may be involved in the pathogenesis of vitiligo. In order to evaluate the free radical scavenger system of vitiligo melanocytes and their susceptibility to oxidative stress, in cultured melanocytes from normal subjects (20) and in cultured melanocytes form patients affected with vitiligo (15) we have evaluated: as enzymatic antioxidants the superoxide dismutase (SOD) and catalase (CAT) activities, as non-enzymatic antioxidants, the intracellular levels of vitamin E (Vit E) and ubiquinone (UBI). The fatty acid pattern of cell membranes as peroxidable compounds. The same parameters and cells viability was evaluated following treatment with cumene hydroperoxide (CUH, 0.1-3 μg/ml) for 1 and 24 h. With respect to normal melanocytes vitiligo melanocytes showed significantly decreased CAT activity (25/45%, $p<0.001$) and, in mean, increased SOD activity (12-25%, $p<0.05$), higher Vit E and decreased UBI levels (35-45%, $p<0.005$), whereas the fatty acid pattern was not significantly different. In normal melanocytes CUH produced 20-40% of toxicity at 3 μg/ml following 24 h treatment. Cultured vitiligo melanocytes were more susceptible to the treatment with peroxidizing agents showing 20-40% toxicity at the lower concentrations used and after 1h treatments. A more pronounced modification of the antioxidants evaluated was observed in vitiligo melanocytes. These results indicate that an imbalance of antioxidant system can be detected in vitiligo melanocytes and that this defect may be correlated with the pathogenesis of the disease.

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MELANOCYTES PRODUCE SUPEROXIDE ANION AND NITRIC OXIDE IN RESPONSE TO LOW DOSES OF ULTRAVIOLET RADIATION. P Valverde, P Manning*, A Graham, CJ McNeil, AJ Thody. Departments of Dermatology & *Clinical Biochemistry, University of Newcastle upon Tyne, UK.

Ultraviolet (UV) radiation excites ground-state molecular oxygen, producing a variety of reactive oxygen species (ROS) which are responsible for many photooxidative and photochemical reactions that damage cells. Because of its exposure to high oxygen tensions and frequent exposure to UV, the skin is vulnerable to oxidative damage. Melanocytes are especially susceptible and one reason is that their low expression of antioxidant mechanisms that exist in keratinocytes and fibroblasts. The purpose of the present study was to determine whether melanocytes may also contribute to oxidative stress by generating reactive radicals such as superoxide anion ($O_2^{\cdot-}$) and nitric oxide (NO). Firstly, we generated $O_2^{\cdot-}$ using the xanthine oxidase/xanthine system (10-100 mU/ml) and monitored its levels by using cytochrome-c immobilized surface modified gold electrodes. This system produced a linear dose-related generation of $O_2^{\cdot-}$ which was considerably reduced in the presence of cultured human fibroblasts or keratinocytes. In contrast, in the presence of human melanocytes or B16 murine melanoma cells, the concentrations of the $O_2^{\cdot-}$ were increased above control levels suggesting that these cells were actually generating $O_2^{\cdot-}$. This was supported in further experiments in which B16 cells, but not human keratinocytes or fibroblasts produced dose-related increases in the $O_2^{\cdot-}$ in response to low doses of UVB, reaching a peak at 20-40 mJ/cm^2 and dropping to control levels at doses $\geq$ 60 mJ/cm^2. The simultaneous monitoring of NO generation by using the iso-NO sensor probe, showed a linear dose-related increase of NO production at all the doses tested (20-160 mJ/cm^2 of UVB). The generation of $O_2^{\cdot-}$ and NO by melanocytes could be significant because these radicals can interact with each other in vivo to produce the highly toxic peroxynitrite and hydroxyl radical. This, together with their low levels of antioxidant enzymes, may help to explain why melanocytes are more susceptible to oxidative damage than keratinocytes or fibroblasts.

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MEDIUM COMPOSITION MAY STRONGLY INFLUENCE MELANIN SYNTHESIS IN CULTURED MELANOCYTES

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There is a growing evidence that cutaneous melanocytes under in vitro conditions alter their melanogenesis pattern, i.e. the ratio between eumelanin and phaeomelanin synthesis. This situation may cause that the result of in vitro experiments cannot be easily intepreted and extrapolated for in vivo conditions. We studied eu- and phaeomelanogenesis in melanocytes isolated from extreme skin phototypes (I and VI) in media with varying concentration of L-tyrosine and L-cysteine. For the analysis of phaeomelanin and eumelanin we utilized a high-performance liquid chromatographic technique and, in addition, we measured sulphur content (as a phaeomelanin marker) in melanosomes with the use of X-ray microanalysis. We showed that the concentration of L-tyrosine and L-cysteine in cultured media have strong influence on type of melanogenesis. Choosing optimal concentration of the two amino acids one may simulate the melanogenesis pattern occuring under in vivo conditions.

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HAIR-BULB AND EPIDERMAL MELANOCYTES DIFFER IN MELANIN PRODUCTION.

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Melanin production and distribution are the main determinants of the skin and hair colour. Although the pigment cells (melanocytes) in epidermis and hair bulbs are of the same origin, they exhibit certain mutual independency. For instance, UV-induced hyperpigmentation, hair greying or the selective destruction of melanocytes in vitiligo usually regard one melanocyte population only. To get a basic idea on qualitative differences in melanogenesis of these two types of pigment cells we measured the sulphur concentration in pigment granules (melanosomes) of epidermal and hair bulb melanocytes from the same individuals. With the use of X-ray microanalysis we were able to show that epidermal melanosomes contained clearly less sulphur (marker of phaeomelanin) than those from hair bulb melanocytes. These differences were observed in individuals with various skin phototypes. Because the contents of sulphur in melanosomes is apparently dependent on the availability of cysteine (glutathione), our future research will focus on the factors responsible for maintaining the intracellular cysteine concentration.

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HUMAN MELANOCYTES USE TISSUE-TYPE PLASMINOGEN ACTIVATOR TO GENERATE PLASMIN AT THE CELL SURFACE

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The proteolytic potential of pigment cells in human skin is poorly appreciated. We have studied human melanocytes grown in a low-serum medium deprived of phorbol esters, cholera toxin and other non-physiological supplements. All parameters were investigated in parallel in cultured human keratinocytes, dermal fibroblasts and two melanoma cell lines. The following assays were done: immunoblotting of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) in cell-conditioned media; ELISA for urokinase receptor (uPAR) in cell extracts; determination of bound plasmin; inhibition of PA activity by monoclonal antibodies; immuno-precipitation of alpha-2 macroglogulin; zymography of secreted matrix metalloproteinases MPP-2 and 9; and a morphologic and dynamic study of collagen type I lattice organization by cast cells.

Human melanocytes secreted tPAand utilized it to generate cell-bound plasmin. No uPA was detected in the cultures but its receptor was found in cell extracts. Both the 72 kDa and 92 kDa MPPs (gelatinases) were secreted in equal amounts. In addition, melanocytes secreted the wide-spectrum proteinase inhibitor alpha-2 macroglobulin. Melanocytes cast into collagen matrices retained a rounded morphology without dendrites and were unable to contract collagen lattices.

Normal human melanocytes are potentially proteolytically active cells with a proteolytic profile distinct from other cutaneous and melanoma cells. This function may pertain to skin physiology in wound healing. Its anomalies may contribute to the pathophysiology of nevus and melanoma.

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EXPRESSION OF THE MOTOR PROTEIN MYOSIN V IN HUMAN NORMAL MELANOCYTES AND MELANOMA CELLS. J.Lambert, J.M. Naeyaert, Dept of Dermatology, University Hospital, Gent, Belgium.

Myosin V is an actin-associated molecular motor represented by the products of the yeast *myo2/myo4* genes, the mouse *dilute* gene and the human *myoxin* gene. Yeast *myo2* gene mutations result in large, unbudded cells that accumulate cytoplasmic vesicles. Mouse *dilute* locus mutations result in coat colour dilution due to adendritic melanocytes and sometimes in neurological deficits and death. The human counterpart of the *dilute* mutation might be found in the autosomal recessive Griscelli-Pruniéras syndrome characterized by partial albinism, silver-blond hair discoloration, primary immunodeficiency and neurological deficits. Skin melanocytes also lack dendrites. These findings suggest that myosin V could be associated with **melanosome transport** and/or formation and function of **dendrites** in melanocytes. We looked at the RNA expression of myosin V in cultured human melanocytes, keratinocytes and fibroblasts and in G361 human melanoma cells by using RT-PCR with primers spanning a 498 bp region of the *myoxin* 3' untranslated region and Northern blots hybridized with a *myoxin* head region cDNA probe. Protein expression was studied in G361 human melanoma cells by indirect immunofluorescence using the anti-*myoxin* monoclonal antibody 9A10 (gift of R.Kennett, Philadelphia). In all cells studied RT-PCR revealed the expected 498 bp cDNA. Northern blot analysis showed 7, 8 and 12 kb transcripts in all the cells, except in the fibroblasts. Melanocytes showed the strongest expression. Strong fluorescence was seen in the perinuclear area and dendrite tips of the melanoma cells. To our knowledge, this is the first demonstration of myosin V expression in human skin cells, particularly in melanocytes.

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NON CULTURED AND CULTURED MELANOCYTES IN THE TREATMENT OF ACHROMIC EPIDERMAL DISORDERS. K. Ongenae, M. De Mil, JM Naeyaert, Department of Dermatology, University of Gent, Gent, Belgium.

Several grafting procedures have been used to induce repigmentation in achromic epidermal diseases such as vitiligo and piebaldism. The use of autologous non cultivated melanocytes was proposed earlier as an easy and cheap method of treatment. Cultivation of melanocytes prior to grafting was also reported to be successful. However controlled studies are lacking and we therefore conducted a controlled study to evaluate both treatment options. Ten patients were included : 5 had stable vitiligo vulgaris in the last two years, 4 had active vitiligo vulgaris and 1 had piebaldism. At day, 1, 12 cm² occipital skin was prelevated superficially. The skin underwent trypsinization overnight to obtain a suspension of keratinocytes and melanocytes. A proportion of the melanocytes was cryopreserved for further culture. The next day, the suspension was seeded on one of two dermabraded symmetrical depigmented areas. Five of the 10 patients underwent the same procedure with cultivated melanocytes in a third area. Four months after grafting the cellular suspension, no significant left-right difference in pigmentation could be observed. In 9 of 10 patients, a blurring of both areas was detected, with a mild erythema or a discrete pigmentation. Within 3 to 4 weeks of treatment with cultivated melanocytes, scattered pigmented patches were observed in the treated third achromic area of all 5 patients. Our controlled study cannot confirm the value of non cultured autologous melanocytes in the treatment of vitiligo an piebaldism. Bilateral blurring at 4 months is due to the dermabrasion. However, grafting cultured melanocytes induced repigmentation. This may be explained by the higher densities of cultured melanocytes at grafting time.

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Rapid Decrease of Phototoxicity after PUVA Bath Therapy

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Administration of 8-methoxypsoralen (8-MOP) in a dilute bath water solution is an effective therapeutic alternative for systemic application of 8-MOP, avoiding systemic side effects such as nausea and cataractogenesis. Although PUVA bath photochemotherapy is now widely used for a variety of dermatoses, standardized guidelines concerning 8-MOP concentration and the interval between bath and UVA irradiation are not yet available. Therefore, the aim of our studies was to determine the optimal time interval between 8-MOP bath and the UVA irradiation as well as the persistence of photosensitivity in normal skin after PUVA bath treatment. Methods: The minimal phototoxicity dose (MPD) was evaluated 72 hours after irradiation in healthy volunteers with no history of skin disease (skin types II-III). The forearms (right or left) were immersed in a 5 mg/l water solution of 8-MOP (temp. 37.5 °C) for 20 minutes. Study I: UVA irradition with doses of 0.25, 0.5, 1, 1.25, 1.5, and 2 J/cm² UVA was performed on 1cm² test sites on the forearms immediately, 20, 40, 60, and 120 minutes after the 8-MOP bath. Result: Irradiation immediately after the 8-MOP bath led to low MPD (0.25-1.25 J/cm² UVA). A sharp increase of the MPD (>2J/cm² UVA) could be demonstrated in the test areas which were irradiated 60 minutes after the 8-MOP bath, thus showing a fast decrease of the 8-MOP activity already after 1 hour. Study II: The test sites were irradiated 1 hour after PUVA bath with doses of 5 and 10J/cm² UVA and 2, 3, and 4 hours after 8-MOP bath with doses from 5/Jcm² up to 30 J/cm² UVA. Result: A further increase of the MPD indicating a rapid decrease of phototoxicity was detected between 1 and 3 hours after the 8-MOP bath. Irradiation 3 hours after the 8-MOP-Bath showed minimal phototoxic doses to be as high as 20-30 J/cm², 4 hours after the bath no phototoxicity could be detected in doses up to 30 J/cm² UVA. Comment: These results indicate, that the optimal time for irradiation is up to 20 minutes after the 8-MOP bath. This leads to relatively uniform and reproducible phototoxic responses. The results of these studies have major implication for PUVA bath therapy. First, the patient has to be irradiated immediately up to 20 minutes after PUVA bath, otherwise the activity of the drug is drastically reduced. Secondly, photosensitivity decreases so rapidly after the 8-MOP bath, that the patient may go on with her/his erveryday life without encompassing restrictions.

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TIME COURSE OF 8-METHOXYPSORALEN ACTIVITY IN PUVA BATH PHOTOCHEMOTHERAPY.

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One limitation of PUVA bath photochemotherapy is the lack of exact guidelines for optimal performance. One issue of major interest is the time course of photosensibilisation of the skin exposed to 8-MOP in a dilute water solution.

Twelve healthy volunteers were exposed to a 20 min. bath in 150 l of an 8-MOP water solution (0.5 mg/L, 37°C). Immediately, as well as 1, 2, 3 and 5 hours after the 8-MOP bath, irradiation was performed with increasing doses of UVA (0.5, 1, 2, 3, 5 J/cm²) on 2 cm² test areas at the back of the volunteers. The minimal phototoxic dose (MPD) was determined 3 days after the UVA exposure. In all volunteers, photosensitivity was highest immediately after the bath with a MPD significantly below 5 J/cm² (0.5-2 J/cm²). One hour after the bath, in all volunteers an erythema could be induced by irradiation with 2 J/cm² up to 5 J/cm². Two hours after the bath, in only 2 volunteers an erythema was inducible by irradiation with 5 J/cm² UVA. Three and 5 hours after the 8-MOP bath no erythema could be induced in any volunteer by irradiation with UVA doses up to 5 J/cm².

These data indicate that for optimal therapeutic effects UVA irradiation has to be administered immediately after the 8-MOP bath. On the other hand, these results imply that after no more than 3 hours after the 8-MOP bath, no strict restrictions on further sun exposure are mandatory, thus allowing the patient to pursue normal life activities.

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PLASMA LEVELS OF 8-METHOXYPSORALEN FOLLOWING BATH-PUVA PHOTOCHEMOTHERAPY.

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Administration of 8-methoxypsoralen (8-MOP) in a dilute bath water solution is an effective therapeutic alternative to oral PUVA therapy, avoiding systemic side effects, offering better bioavailability of the psoralen and requiring much smaller amounts of UVA for induction of therapeutic effects. To obtain exact data about the percutaneous absorption of 8-MOP during a psoralen bath, the plasma levels of the drug were determined in 26 patients with different skin diseases by a reverse high-performance liquid chromatographic method. 15 patients receiving oral PUVA therapy (5 mg 8-MOP/kg body weight) served as a positive control group. Bath solutions were prepared by diluting 15 ml of 0.5% stock solution of 8-MOP in 150 L of bath water (0.5 mg/L, 37°C).

Blood samples were drawn from the patients 5, 30, 60, 120 and 180 minutes after the bath. In the oral PUVA group blood samples were obtained 1½ hours after administration of the drug.

In 23 of 26 patients, 8-MOP levels were undetectable in every blood sample. After 30 minutes, two patients showed detectable levels of 8-MOP (5 ng/ml, 7 ng/ml), while 60 minutes after the PUVA bath 8-MOP was detectable in only one volunteer (5 ng/ml). In patients receiving oral 8-MOP therapy, serum levels varied between 45 and 360 ng/ml 1½ hours after drug administration.

Our data confirm extremely low 8-MOP levels resulting from 8-MOP bath water treatments and provide confirmation of the absence of systemic side effects in patients who are undergoing PUVA bath therapy.

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INCREASED CYTOKINE PRODUCTION, BUT DECREASED T CELL PROLIFERATION FOLLOWING PUVA. Matthias Lüftl, Dagmar Dick, Gerd Plewig, Martin Röcken, Department of Dermatology, Ludwig-Maximilians-University, Munich, Germany.

Photochemotherapy using 8-methoxypsoralen (8-MOP) and UVA (PUVA) is an established therapy for a large spectrum of T cell mediated skin diseases, such as psoriasis, lichen planus or cutaneous T cell lymphoma. However, the exact mode of action of PUVA in these diseases remains unknown. Here, we analyzed the effects of a single PUVA treatment on T cells, using long term cultured cell lines (CTLL) and concanavalin A (ConA) induced T cell blasts derived from freshly isolated polymorpho nuclear blood lymphocytes. Day 10 ConA blasts were incubated with 100 ng/ml of 8-MOP, irradiated with increasing doses of UVA (0.1-2.0 J/cm2), restimulated 0-48h after PUVA with ConA, and subsequently analyzed for proliferation or for cytokine production. After PUVA treatment with > 0.5 J/cm2, viability of the T cell blasts dropped to 50% (of the controls), and mitogen as well as IL-2 driven T cell proliferation were reduced by > 90%. FACS analyses revealed, that CD8 and CD4 T cells were equally reduced in number. When restimulated directly after PUVA the supernatants of mitogen stimulated T cells contained rather increased levels of IL-2 and IFN-γ (2-10 fold) as compared to untreated, 8-MOP or UVA treated populations. Since mitogen induced transcription of IL-2 mRNA was also rather increased than decreased after PUVA, the data suggest that PUVA causes inhibition of T cell proliferation, but not of cytokine production or cytokine gene transcription. Thus, PUVA might influence T cell mediated diseases not only via UVA induced psoralen-DNA cross links but also by its effect on the local cytokine milieu.

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PUVA INTERFERES WITH REPLICATION, BUT NOT TRANSCRIPTION, AT SUBLETHAL PSORALEN CONCENTRATIONS AND UVA DOSAGES. Matthias Lüftl, Claudia Kammerbauer, Gerd Plewig, Martin Röcken, Klaus Degitz, Department of Dermatology, Ludwig-Maximilians University, Munich, Germany.

Photochemotherapy using psoralens and UVA radiation (PUVA) is an established treatment for many skin disorders. Psoralen-DNA interactions as well as PUVA-induced reactive oxygen intermediates (ROI) might account for the biologic effects of PUVA. Antiinflammatory and antiproliferative effects of PUVA might be caused by inhibition of replication and/or transcription. We have investigated the effects of single applications of PUVA on both replication or transcription in a promyelocytic (HL60) and a keratinocyte (HaCaT) cell line. Proliferation (measured as H3-thymidine incorporation) was inhibited by PUVA in both cell lines. The inhibition increased with increasing 8-methoxypsoralen (8-MOP) concentrations or UVA dosages and was effective at conditions not affecting cell viability up to 48h after PUVA. Since PUVA induced DNA modifications might not only interfere with replication, but also with transcription at promoter sites, we studied the effect of PUVA on the transcription of ICAM-1 and c-Jun. In HL60 cells, both low constitutive and high IFN-γ induced ICAM-1 mRNA expression was not influenced by PUVA (500 ng/ml of 8-MOP; 0.25 J/cm^2 UVA). Similarly, low constitutive and high phorbol ester induced c-Jun mRNA expression was not inhibited by PUVA. These findings suggest that PUVA causes inhibition of proliferation, but not gene transcription, and that interference with replication may be the primary therapeutic effect of UVA induced psoralen-DNA cross links. In order to assess possible transcriptional effects of PUVA generated ROI, the ROI sensitive transcription factor NFκB was assayed in mobility shift assays. NFκB-specific binding activity was not induced 1 to 24h after PUVA (500 ng/ml of 8-MOP; 1.0 J/cm^2 UVA) in extracts from PUVA treated HaCaT cells when compared to untreated controls, while the pro-oxidant TNF-α did cause a marked increase in NFκB-specific binding after 1h. These data do not support a major role for PUVA generated ROI in transcriptional regulation.

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PUVA treatment inlduces an oxidative damage in murine lymphoma cells.

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Photochemotherapy employing 8-methoxypsoralen (8-MOP) in association with long wavelength UV irradiation (PUVA) makes use of the photoxicity and covalent photo-addition of photosensitizer with DNA. In several studies performed during the last years, however, it has been demonstrated that PUVA induces changes in a large number of subcellular sites, i.e., proteins and lipids, due to the generation of reactive oxygen species. In order to establish the role of cell membranes as potential target of PUVA-induced cell toxicity, we have investigated the modification of fatty acid pattern of cell membranes and intracellular level of Vitamin E (Vit E) (by gas-chromatography mass spectrometry) in a murine lymphoma cell line RMA-S. 15 x 10^6 cells were treated with 50, 100 and 200 ng/ml of 8-MOP and subsequently exposed to 1J/cm^2 UVA and kept in culture medium without fetal calf serum at 37°C for several hours. Analyses performed at time 0 demonstrated, in comparison to non-treated controls, a 30% reduction of Vit E level in the UVA alone treated cells. In the 8-MOP/UVA treated cells a 45-75% decrease of Vit E levels, correlated to the 8-MOP doses, was found. A significant decrease of the percentage of polyunsaturated fatty acids (PUFA) was detected in PUVA-treated cells (25-35%). After 6 h of culture both Vit E level and PUFA percentage showed a further decrease of 3-5 fold in respect to controls. These data suggest that the reduction of Vit E concentration and the oxidation of cell membrane lipids are dramatically evident in the period immediately following 8-MOP/UVA treatment and may play a role in the biological modifications induced by PUVA therapy.

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PUVA (8-METHOXYPSORALEN + UVA) TREATMENT ENHANCES FIBROBLAST DIFFERENTIATION ALONG A TERMINAL PATHWAY. G.Herrmann (1,2), M.Wlaschek (1), P.Brenneisen (1), J.Wenk (1), E.Schubert (1), Th.Krieg (1), G.Goerz (2), K.Scharffetter-Kochanek (1), Dept. Dermatol., Univ. Cologne (1), Dept. Dermatol., Univ. Düsseldorf, (2) FRG

Recently, patients with localized scleroderma have successfully been treated with 8-methoxypsoralen (8-MOP) plus UVA (PUVA). The molecular basis for this successful treatment is unknown. Since matrix-metalloproteinases (MMP) might be responsible for the resolution of the sclerotic plaques we addressed the questions (1) whether PUVA treatment of fibroblasts results in MMP induction, (2) whether reactive oxygen species might be involved, and (3) whether the inhibitor of MMPs, TIMP-1, is also affected. Preincubation of fibroblasts with 50 ng/ml 8-MOP and irradiation at a dose of 90 kJ/m^2 (UVASUN3000, 340-450 nm, Mutzhas, Munich) only marginally affected protein synthesis and cell viability. However, treatment of fibroblasts resulted in a permanent switch of mitotic to postmitotic fibroblasts suggesting that PUVA treatment may promote aging of fibroblasts along a terminal differentiation pathway. A 2-fold increase in MMP-1 (collagenase) and MMP-3 (stromelysin-1) mRNA occured at 24 h after UVA or PUVA treatment. At 48 h after UVA alone MMP mRNA levels had returned to basal levels, whereas a further increase in MMP mRNA was detected after PUVA remaining up-regulated over 120 h. In contrast to the UVA induction of MMPs, singlet oxygen could not be identified as an intermediate in the response to PUVA. TIMP-1 mRNA was only marginally induced following PUVA. Similar results were obtained on protein level. Our data show that PUVA treatment leads to an enhanced and substantially prolonged induction of MMPs. The imbalance between MMPs and their inhibitor TIMP-1 most likely contributes to connective tissue degradation finally resulting in the resolution of sclerotic plaques in fibrotic skin disorders.

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8-METHOXYPSORALEN PLUS UVA LIGHT INDUCES APOPTOSIS IN RMA-S CELLS

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The clinical efficacy of photochemotherapy employing 8-methoxypsoralen (8-MOP) in association with long wavelength UVA (PUVA) light has been attributed in the past to the adduct formation of photosensitized psoralens with DNA. In several studies performed over the last ten years the biological effects seem to range from a modification of immunogenicity to frank cytotoxicity of treated cells. In order to establish the mechanism of cell death induced by PUVA or photophersis (EcP) we investigated the involvment of apoptosis (programmed cell death) in RMA-S cells, a murine lymphoma cell line. Cells treated with 10-,50-,100-,300 ng/ml of 8-MOP and 1J/cm^2 UVA generated a 4-, 11-,47-,73 % reduction of cell viability, respectively, after a 24 hours cell culture period at 37°C, whereas the same treatment and subsequent cell culture at subphysiological temperatures (26°C) did not, underlining the role of physiological temperatures in the induction of cell death. Flow cytometry analysis demonstrated a modification in the morphology of 300ng/ml 8-MOP+ 1J/cm^2 UVA treated RMA-S cells consisting in the reduction of cell volume and increase of cell density in part of the phototreated cell population. In addition, the DNA fragmentation was quantified by staining with propidium iodide showing an significant increase of apoptotic bodies in the PUVA treated cells after 24 hours in comparison to controls. The induction of apoptosis alone , however, could not explain the massive cell death induced by PUVA, indicating two seperate mechanisms of cell death, apoptosis and necrosis. The molecular events favouring one or the other possible mechanism remain still to be established. However, these data suggest that the induction of cell death in 8-MOP/UVA treated cells may play an additional role in the therapeutic effects observed in patients treated with PUVA or EcP photochemotherapy.

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ERYTHEMA- WEIGHTED DOSAGE IN DERMATOLOGICAL PHOTOTHERAPY

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Both wanted and unwanted effects of phototherapy strongly depend from the spectral distribution of the actual UV source. In terms of efficiency, most of the known action spectra show a steep decrease by a factor of approximately 10^3 between the wavelengths 300 to 330 nm. In this region the graph of erythema action, psoriasis clearing, photocarcinogenesis and skin photoaging is quite similar.

Radiation sources used for dermatological phototherapy can be classified as type "UV-A/B" (285-385 nm) or "UV-A" (315-385 nm). Most of "UV-A/B" devices not only emit UV-A and UV-B-1 (295-315 nm) but also not negligible amounts of UV-B-2 (285-295 nm) and in some cases even UV-C (200-285 nm).

Because of the 10^3 decrease and the broad-band emission of phototherapeutic equipment, the usual description in J/cm^2 just poorly reflects UV efficiency. Therefore, the spectral irradiance of 10 devices often used in UV-A/B phototherapy was monitored between 280 and 400 nm (resolution 1 nm) and folded with the standard CIE erythema action spectrum. This resulted in erythema-weighted data ($J_{erythema}$/cm^2) characterising the actual phototherapeutic device.

This way, (1) minimal erythema dose can be estimated with a small diagnostic device, followed by calculation of an optimal starting dosage for any given UV treatment device. Furthermore, (2) erythema efficiency of different equipment can be evaluated, allowing (3) the safe transposition of the patient to another UV device. (4) Biological significance of cumulative dosages received on different devices is comparable.

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INTERLEUKIN 12 BREAKS UV-INDUCED TOLERANCE BY AFFECTING RATHER CD8+ SUPPRESSOR CELLS THAN CD4+ EFFECTOR CELLS. Agatha Schwarz, Stephan Grabbe, Yoshinori Aragane, Kirsten Sandkuhl, Helge Riemann, Thomas Luger, Giorgio Trinchieri*, and Thomas Schwarz, Ludwig Boltzmann Institute for Cellbiology and Immunobiology, Department of Dermatology, University Münster, Münster, Germany, *The Wistar Institute, Philadelphia PA

Since we recently demonstrated that intraperitoneal (i.p.) injection of interleukin (IL)-12 breaks UV-induced tolerance, we were interested in the underlying mechanisms. C3H/HeN mice were sensitized with dinitrofluorobenzene (DNFB) through UVB exposed skin and ear challenge performed 5 d later. UV-treated mice resensitized 14 d after the first challenge through non-UV-exposed skin displayed hapten-specific tolerance, whereas UV-exposed mice injected i.p. with IL-12 before *re*sensitization produced a significant ear swelling response confirming that IL-12 can break UV-induced tolerance. Whereas adoptive transfer of spleen cells from UV-irradiated mice inhibited sensitization of recipient mice, no inhibiton was observed after transfer of spleen cells from UV-exposed and IL-12 treated mice. Depletion of either CD4$^+$ or CD8$^+$ T-cells revealed that UV-induced suppression is transferred via CD8$^+$ cells. To prove whether IL-12 overcomes tolerance by inhibiting CD8$^+$ suppressor cells or by activating CD4$^+$ effector cells, splenocytes from UV-exposed, DNFB treated and IL-12 injected mice were depleted from CD4$^+$ cells and transferred into naive mice which were subsequently sensitized. Transfer of CD4 depleted splenocytes from UV-irradiated and IL-12 treated mice still resulted in lack of suppression of sensitization in recipients. The same outcome was observed when UV-exposed donor mice were treated with IL-12 before *re*sensitization. Thus, these data suggest that IL-12 breaks UV-induced tolerance by acting rather on CD8$^+$ suppressor than on CD4$^+$ effector cells.

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MOLECULAR MECHANISMS INVOLVED IN UVB-MEDIATED SUPPRESSION OF INTERFERON γ-INDUCED EXPRESSION OF INTERLEUKIN-12 P40 IN HUMAN MONOCYTES. Yoshinori Aragane, Agatha Schwarz, Karin Große-Heitmeyer, Birgit Pöppelmann, Akira Maeda, Thomas Luger, and Thomas Schwarz. Ludwig Boltzmann Institute for Cellbiology, Department of Dermatology, University Münster, Münster, Germany

One of the most important immunosuppressive properties of UV-light is its ability to suppress the induction of contact hypersensitivity (CHS). Recently, we could show that interleukin (IL)-12 is critically involved in CHS and that injection of IL-12 can reverse this type of UV-induced immunosuppression. Thus, we were interested to investigate whether UVB influences the gene expression of IL-12. Upon treatment with interferon γ (IFNγ) human monocytes isolated via plastic adherence reacted with a marked induction of IL-12 p40 mRNA determined by northern blot analysis, while simultaneous exposure with UVB abolished IFNγ-induced p40 mRNA expression significantly, indicating that UVB suppresses IL-12 p40 mRNA expression induced by IFNγ. To further elucidate the molecular mechanisms involved, we next focused on the currently cloned-promoter region of the IL-12 p40 gene. Therefore, bandshift assays were performed using oligonucleotides encoding for recognition sequences of AP-1, NF-IL-6, NFκB, SP1, and IRF-1 all derived from the IL-12 p40 promoter. While IFNγ failed to enhance the binding of AP-1, NF-IL-6 and SP-1, respectively, the binding of NFκB and of IRF-1 were markedly induced by IFNγ, suggesting that the IFNγ-induced binding of these two transcription factors may be crucial in IFNγ-induced IL-12 p40 expression. Furthermore, the simultaneous UVB exposure significantly interfered with the binding of NFκB and IRF-1 both activated by IFNγ treatment. These data demonstrate for the first time that UVB suppresses IFNγ-induced expression of IL-12 p40 through the interference with transcriptional activators.

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DOSE DEPENDENT IMMUNOSUPPRESSION, P53 INDUCTION AND APOPTOSIS IN SKIN EXPLANTS FOLLOWING UV IRRADIATION. V Davenport, JF Morris, H Mehmet and AC Chu. Royal Postgraduate Medical School, London, UK.

To examine the relationship between UV irradiation and the development of skin cancer, we have investigated the dynamics of UVB induced immunosuppression, p53 expression and apoptosis in human skin explants.

Human skin explants were irradiated with a range of pure UVB doses (47 $mJcm^{-2}$ to 1.4 Jcm^{-2}), using a TLO1 sunlamp. Part of each explant was then used to generate stimulator cells for a mixed epidermal cell lymphocyte reaction, used to measure Langerhans' cell (LC) function. The remainder of the explants were cultured for 18-24 hrs, in 10% serum, at 37°C, then a sample of each was paraffin embedded and stained for apoptosis by in situ end labelling. P53 protein levels were quantified in epidermal cell lysates, prepared from the remaining fractions, by western blotting.

A UV dose dependent reduction in LC function and a concomitant increase in p53 protein expression was observed. Similarly, a dose dependent increase in apoptosis was found in the skin explants following UVB irradiation, ranging from 7 cells mm^{-2} in controls to 244 cells mm^{-2} following a dose of 705 $mJcm^{-2}$; however, at UV doses higher than this apoptosis decreased in a dose dependent manner.

These results indicate a close correlation between UVB induced epidermal immunosuppression (shown by reduced LC function), DNA damage (indicated by p53 protein induction) and cell death by apoptosis (determined by ISEL). The precise relationship between immunosuppression, p53 expression and apoptosis following UV irradiation is under current investigation.

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THYMINE DIMER FORMATION IS CAUSALLY RELATED TO ULTRAVIOLET B RADIATION (UVBR)-INDUCED IMMUNOSUPPRESSION IN VIVO IN HUMAN SKIN. H. Stege, L. Roza*, and J. Krutmann, Dept. Dermatol., Univ. Düsseldorf, Germany; *TNO Nutrition & Food Res., Dept. Genetic Toxicol., Rijswijk, The Netherlands.

We have previously demonstrated that i.c. injection of rh IFN-γ into human skin upregulates keratinocyte (KC) expression of intercellular adhesion molecule-1 (ICAM-1), and that IFN-γ-induced KC ICAM-1 expression is suppressed, if IFN-γ is injected into a skin area which has been exposed to UVBR (1 MED) prior to IFN-γ stimulation. This model system was now further employed to assess the potential role of DNA photoproducts in UVBR-induced immunosuppression in vivo in human skin. When biopsies from buttock skin of healthy human volunteers (n=9) were analyzed, UVBR-induced suppression of KC ICAM-1 expression was found to be associated with the formation of thymine dimers, as was assessed in the same biopsies by quantitative immunofluorescence microscopy employing the thymine dimer specific mAb H3. In order to determine the functional relevance of thymine dimer formation, irradiated human skin was treated with liposomes containing the DNA repair enzyme photolyase (Photosomes®), which specifically removes thymine dimers from cellular DNA if the enzyme/DNA complex is exposed to photoreactivating light. Treatment of human skin (n=6) immediately after UVBR exposure with Photosomes® plus photoreactivating light reduced the number of thymine dimers in epidermal cells by approximately 45 %. Photolyase-induced removal of thymine dimers from UVB-irradiated human skin was of functional relevance, since UVBR-induced suppression of IFN-γ-induced KC ICAM-1 expression was prevented within these skin areas. We have demonstrated that topical application of photolyase to human skin is effective in providing DNA repair and immunoprotection. Our studies indicate that thymine dimer formation is relevant for in vivo UVBR-induced immunosuppression in human skin.

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SUNSCREEN PROTECTION AGAINST UVB-INDUCED IMMUNO-SUPPRESSION IN HUMANS. HMH Hurks, C Out-Luiting, RG van der Molen, BJ Vermeer, FHJ Claas and AM Mommaas, Depts of Dermatology and Immunohaematology and Bloodbank, Laboratory for Electron Microscopy, University Hospital Leiden, Leiden, The Netherlands.

Ultraviolet (UV) radiation has been shown to suppress the (skin) immune system. Whether sunscreens can prevent immunosuppression is a matter of debate. This study investigated the protective capacity of a commercial sunscreen lotion in humans. Part of the right arm of healthy volunteers was exposed to a erythemagenic UVB dose of 160 mJ/cm^2 for 4 consecutive days. Before irradiation, sunscreen was applied on the skin or on a piece of quartz (to avoid penetration of the sunscreen below the stratum corneum where trans urocanic-acid (UCA) can be isomerized to cis-UCA). The control group was irradiated without prior application of sunscreen. Four hours after the last irradiation, epidermal sheets were obtained by the suction-blister method from both arms and epidermal cells were used as stimulator cells in the mixed epidermal cell lymphocyte reaction (MECLR). Responses were expressed as percentages of the non-irradiated left arm. The MECLR responses in the control group were significantly increased (205%). This increase was correlated with an expansion of $CD36^+DR^+$ macrophages, known to be involved in the induction of T suppressor cell mechanisms. Application of sunscreen before UVB exposure, either on a piece of quartz or on the skin, prevented the increase of $CD36^+DR^+$ cells completely. When the sunscreen was applied on the piece of quartz, MECLR responses of 98% were observed. However, when sunscreen was applied on the skin, the MECLR responses were slightly, but significantly decreased to 85%, showing a good, but not complete protection against UVB-induced immunosuppression. The same sunscreen was tested in an earlier study, where volunteers were exposed thrice weekly to suberythemagenic doses of UVB during 4 weeks. MECLR responses were decreased to 20% and the sunscreen was not able to prevent this suppression, not even partly. These contradicting results indicate that the UV treatment (e.g. erythemagenic or not, long or short-term) can be determining how effective sunscreens can protect against UV-induced immunosuppression.

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PROTECTIVE POTENCY OF TOPICAL SUNSCREENS AGAINST UV-INDUCED IMMUNOSUPPRESSION. T. Neubert, B. Homey, H.-C. Schuppe, H.-W. Vohr*, T. Ruzicka, P. Lehmann. Dept. of Dermatology and *Institute of Genetics, University of Düsseldorf, D-40233 Düseldorf.

Ultraviolet (UV) radiation causes sunburn, immunosuppression and photocarcinogenesis. We evaluated the immunosuppressive effects of solar simulated irradiation (SSR) and compared the protective capacity of UVB and UVA/UVB filter containing sunscreens in a murine local lymph node assay. Hairless C3H mice were irradiated with increasing doses of SSR with and without sunscreen for six consecutive days. On day 7-9, mice were sensitized with oxazolone on the dorsal surfaces of both ears and the draining auricular lymph nodes (LN) were removed on day 10. Irradiation with SSR of mice treated with placebo and oxazolone resulted in decreased LN-cell counts, $CD11b^+/Ia^+$ and $CD4^+/CD69^+$ LN-cell subpopulations when compared with unirradiated and oxazolone sensitzed mice. Sunscreen application could partially protect against UV-induced immunosuppressive effects. Both sunsreens did not show significant differences in their potency to protect against UV-induced immunosuppression. In addition, a minimal immunosuppressive dose (MISD) and a minimal UV-induced edema dose (MEdD) with and without sunscreen was defined. Comparing these parameters, the MISD was significantly lower than the MEdD for both sunscreens. In conclusion, UVB and UVA/UVB filter containing sunscreens exerted some protection against UV-induced immunosuppression. However, this protective capacity was less effective than the potency to prevent UV-induced edema.

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IMMUNOSUPPRESSION INDUCED BY ACUTE SOLAR-SIMULATED ULTRAVIOLET (UV) EXPOSURE IN HUMANS: PREVENTION BY A LOW SUN PROTECTION FACTOR (SPF) SUNSCREEN. I. Serre, J.P. Cano, J. Meynadier* and L.Meunier*. Laboratory of Drug Toxicology and Department of Dermatology*, University of Montpellier, France.

UVB exposure reduces immunization rates to epicutaneous antigens in humans and recent data suggested that high SPF sunscreens prevent UVB-induced immunosuppression. The purpose of our study was to evaluate the protective effect of a low SPF sunscreen on the reduction of contact hypersensitivity responses that occurs in humans after an acute solar-simulated irradiation. After initial dinitrochlorobenzene (DNCB) sensitization (30μg) on the buttock, the elicitation was performed on unirradiated upper inner arm skin with a panel of four different doses of DNCB (3, 6, 9 and 12μg). The skin fold thickness was determined to score the elicitation phase and the irritating effects of DNCB. For each site, the mean increase of skin thickness due to the irritating effects of DNCB was subtracted from the increase in skin thickness due to the elicitation response. Sensitization on UV-irradiated sites was performed 3 days after a 3 MED UV-exposure (Dermolum solar simulator equipped with a WG 320 filter) and a SPF 15 sunscreen formulation, containing a combination of Eusolex 232, Uvinul N539, Parsol 1789 and Mexoryl SX, was applied on the buttock to be irradiated. Upon recruitment each of male volunteers (n = 147) was randomly assigned into one of 8 groups (Gr): sensitization and elicitation with (Gr A-UV, n = 20) and without UV exposure (Gr A, n = 20); elicitation with (Gr B-UV, n = 14) and without UV (Gr B, n = 19); sunscreen application prior to sensitization and elicitation with (Gr C-UV, n = 20) and without UV exposure (Gr C, n = 17); elicitation after sunscreen application on a buttock with (Gr D-UV, n = 20) and without UV exposure (Gr D, n = 17). The A-UV group had a reduced response rate to challenge doses of DNCB compared with Gr A ($p \leq 0.005$) and Gr C-UV ($p \leq 0.015$). Gr A, Gr C and Gr C-UV showed no significant differences in responses rate to any of the doses of DNCB tested. The mean increases in skin thickness due to challenge reactions were similar in control groups (Gr B, Gr B-UV, Gr D and Gr D-UV). In conclusion, combinations of sunscreens with low SPF but effective UVA protection may adequately prevent the suppression of contact hypersensitivity induced by an acute solar-simulated irradiation.

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EVALUATION OF THE EFFECT OF SOLAR SIMULATOR RADIATION ON CONTACT HYPERSENSITIVITY IN MAN AS A RISK FACTOR FOR SKIN CANCER.

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Ultraviolet radiation (UV) is both mutagenic and immunosuppressive. We investigated the effects of Solar Simulator Radiation (SSR) on the contact hypersensitivity response in normal and skin cancer patients compared to unirradiated control subjects.

16 healthy volunteers and 16 patients with at least one histologically proven non melanoma skin cancer were irradiated with 3 MED SSR on buttock skin and immediately sensitised with a 2% solution of Diphenylcyclopropenone on this site. 7 healthy volunteers were similarly sensitised on unirradiated buttock skin. Contact hypersensitivity was elicited at one month on unirradiated, sun protected arm skin using serial challenge concentrations of DPCP (10^{-5} to 1%).

The results show that all patients elicited contact hypersensitivity though at variable challenge concentrations. 3 / 16 (19%) irradiated normals and 9 / 14 (64.3%) irradiated skin cancer patients required a higher challenge concentration to elicit a response compared to the unirradiated control group. There was a significant difference in the responses between the irradiated normals and skin cancer patients, p=0.02 (Fisher's Exact Test). The mean challenge concentration required to elicit a response was also higher in the skin cancer group compared to the irradiated normals, p=0.02, and unirradiated controls, p=0.004 (Mann-Whitney Test).

A single dose of SSR followed by immediate sensitisation suppresses contact hypersensitivity, susceptibility to which may be a risk factor for skin cancer.

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UVA EXPOSURE OF HUMAN SKIN DOES NOT INHIBITS THE INDUCTION OF CONTACT SENSITIVITY IN CONTRAST TO UVB EXPOSURE.

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In humans UVB-exposure of the skin reduces immunization rates to epicutaneous antigens. Since we are exposed to increasing amounts of UVA radiation due to altered social behaviour and use of UVA-tanning salons, it is important to determine whether UVA irradiation reduces the immunization rates. Forty-eight volunteers were assigned to receive no sensitization, sensitization with diphenylcyclopropenone (DPCP) on non-UV-exposed normal skin or sensitization with DPCP on skin exposed to three minimal erythema doses (MED) of either longwave UVA or UVB radiation 3 days prior to sensitization. Three weeks after sensitization all volunteers were challenged with five different concentrations of DPCP. The challenge reactions were scored clinically and the increase in skin thickness was measured using a micrometer. Confirming previous reports sensitization on UVB-exposed skin resulted in a reduced immunization rate since only 33% had a positiv clinical challenge reaction (n=15) compared to sensitization on non-irradiated skin where 75% had a positiv challenge reaction (n=12). The reduced immunization rate result in a marked reduced increase in the skin thickness in the UVB group (0.54 ± 1.11mm) compared to the control group (2.04 ± 1.74mm) ($p<0.03$). In contrast, sensitization on skin exposed to 3 MED UVA radiation did not result in a decreased immunization rate since 85% had a clinical positive challenge reaction (n=13). Sum increased in skin thickness after challenge in the UVA group (2.54 ± 2.32mm) compared to the control group (2.04 ± 1.74mm) ($p>0.6$). These results demonstrate differential effect of UVB and UVA irradiation on the skin immune system and may in part explain the less carcinogenic effect of UVA irradiation.

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THE PHOTOPROTECTIVE EFFECT OF REPAIR ENZYMES ON P53 INDUCTION IN SKIN EXPLANTS

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This study aimed to elucidate the photo-protective effects of photorepair and photoreactivating enzymes applied to skin explants pre and post irradiation, using p53 protein as marker of cellular damage.

Test preparations P-T included repair enzyme T4N5 and a photo reactivating enzyme extracted from *Anacystis nidulans*, either in aqueous solution or 15% dilution in a gel base and gel base alone. P-T and a PBS control were standardly applied to human skin explants ($6cm^2$) at $2\mu l/cm^2$, and incubated for 1 hour at 37°C either prior to or directly after irradiation with a range of UVB doses ($37mJcm^{-2}$ to $444\ mJcm^{-2}$), using a filtered FS20 sunlamp. Treated explants were decontaminated then cultured for 18-24 hrs at 37°C, 5%CO_2. Epidermis was enzymatically isolated and P53 separated from lysates of $5x10^6$ epidermal cells per treatment, using polyacrylamide gel electrophoresis, then identified using Western blotting and DO-1, mouse anti-human monoclonal antibody.

Fifty percent reductions in p53 levels were detected in explants treated with either enzyme in gel base, applied both pre and post irradiation. Application of the more concentrated enzymes in solution post irradiation, resulted in stimulation of p53 above control levels at the lower UV doses.

Photoreactivating and photorepair enzymes may prove beneficial additions to both sunscreens and aftersun lotions by helping to reduce epidermal damage at the DNA level.

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THE PHOTOIMMUNOLOGICAL PHENOTYPE OF HUMAN CELLS IS DETERMINED AT THE LEVEL OF DNA-REPAIR-ENZYME FUNCTION.

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We have previously demonstrated that cells from Xeroderma pigmentosum group D (XP-D) patients, which are both DNA repair defective and skin cancer prone, are more susceptible to ultraviolet B radiation (UVBR)-induced immunosuppression than cells from trichothiodystrophy (TTD) patients, which are also DNA repair-defective, but do not develop skin cancer. Both syndromes are based on mutations within the XPD gene. The XPD protein does not only serve as a DNA repair enzyme, but also as a transcription factor. Mutations within the XPD gene may therefore result in defective DNA repair, but also cause an altered phenotype with regard to UVBR-induced transcriptional control of immunologically relevant genes such as ICAM-1. In order to test whether the abnormal photoimmunological phenotype of XP-D cells is determined by the defect in the XPD gene, we used a retro-viral vector to transfer a functional copy of the XPD cDNA into cells from an XP-D patient. We have found that transfection of XP-D cells with the XPD gene corrected their abnormal photoimmunological phenotype. In untransfected XP-D cells, UVBR suppressed IFN-γ mediated ICAM-1 mRNA expression in a dose-dependent manner with a half-maximal effect at 20.8 J/m^2. In XP-D cells transfected with and complemented by the XPD gene, this dose-response was shifted towards significantly higher UVBR doses with an ED_{50} of 46.2 J/m^2, which did not differ from that observed in normal cells (ED_{50} = 48J/m^2). Transfection of XP-D cells with the XPC gene did not alter their photoimmunological phenotype, although efficient integration, mRNA and protein expression was obtained. We propose that DNA repair enzyme functions determine the susceptibility of a human cell towards UVBR-induced immunosuppression.

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KNOCKOUT MICE HETEROZYGOUS FOR THE *XERODERMA PIGMENTOSUM A* GENE DO NOT HAVE AN INCREASED SUSCEPTIBILITY TO UVB CARCINOGENESIS

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Clinically normal carriers of *xeroderma pigmentosum* genes (heterozygotes) are much more common in the general population than are patients (homozygotes). Recently established transgenic mice with a deficiency in the *xeroderma pigmentosum* group A (*XPA*) gene can serve to establish whether such heterozygotes have an increased skin cancer susceptibility. Therefore, *XPA* knockout mice (129/ola-C57Bl/6) were crossed with albino hairless mice (HRA:SKH), and the hairless offspring (an F2 intercross) was daily exposed for 6 min/day to UV radiation from F40 sunlamps: Fourteen mice of each genotype (*XPA*-/-, *XPA*+/-, and *XPA*+/+) to a daily dose of 80 J/m^2 (250-400 nm). In contrast to the *XPA*+/- and the *XPA*+/+ mice, the *XPA*-/- mice showed dryness, scaling, and redness of the exposed skin preceding the appearance of skin tumors. Tumor induction times (median times, t_{50}'s) were determined after exclusion of frank papillomas from the data. The t_{50}'s for tumors with a diameter of 1mm for *XPA*-/-, +/-, and +/+ mice were 78 days, 316 days, and 337 days, respectively. The difference between the +/- and the +/+ group was statistically not significant. Hence, no increased susceptibility in heterozygotes is detected, and complete loss of functional *XPA* genes results in a decrease in latency time by about a factor four.

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INCOMPLETE DNA REPAIR AFTER UVA_1 BUT NOT AFTER UVB OR PUVA

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The time course of DNA repair was investigated using the nucleoid sedimentation technique. We irradiated human lymphoid cells with equilethal doses (approx. a percentage of 50) of UVB, UV-A/B, UVA and UVA1 and used the fraction of cells without lethal damage to cytoplasmatic membrane in our investigations. Additionally PUVA treatment of cells using two different light sources was performed. As results we found in case of UVB and UV-A/B irradiation of the cells a DNA repair activity that lasts not longer than 4 hours, whereas in case of UVA and UVA1 no repair activity was detectable but an increasing nucleoid density that is supposed to indicate apoptosis. There was no repair activity up to 24 hours when the irradiation dose of UVA and UVA1 was lowered to one fifth of the equilethal dose. There was no difference between UVA and UVA1 in the nucleoid density time course. In case of PUVA we found a DNA repair that is completed after 12 hours. An UVB part in the irradiation spectrum of a PUVA system predominates the repair kinetics. This is thought to be an effect of blocked binding sites for the 8-MOP due to pyrimidine dimer formation and of the lower structural complexity of UVB damage that requires simpler repair pathways. Our results show the benefit of time course studies of the DNA repair: short time repair, incomplete repair, apoptosis depending of the irradiated wavelength.

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ULTRAVIOLET B RADIATIONS DOWN REGULATE NERVE GROWTH FACTOR mRNA AND RELEASE IN HUMAN KERATINOCYTES. Alessandra Marconi, Caterina Chiodino, Cristina Vaschieri, Alberto Giannetti and Carlo Pincelli
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Nerve Growth Factor (NGF) is synthesized and released by human keratinocytes. NGF has been recently regarded as a pro-inflammatory cytokine. Furthermore, NGF stimulates the proliferation of human keratinocytes and NGF levels are increased in inflammatory cutaneous conditions, such as psoriasis. Because ultraviloet B radiations (UVB) modulate the expression of many epidermal cytokines, the purpose of the present study was to evaluate the UVB effects on NGF mRNA expression and release in normal human keratinocytes. Keratinocytes were cultivated in serum-free medium (KGM) or on a feeder-layer of 3T3 cells, which were either lethally irradiated or mitomicin-treated. At sub-confluency, keratinocytes were irradiated with increasing UV-B doses (10, 25, 50 mJ/cm^2). Keratinocyte conditioned media were collected at different times and analyzed by an ELISA assay specific for the active biological form of NGF (β-NGF). NGF mRNA from keratinocytes was evaluated by reversetranscription-polymerase chain reaction (RT-PCR). UVB induced a significant and dose-dependent decrease in NGF levels in keratinocyte supernatants ($p \leq 0.002$) from 24 to 72 hrs. In addition, NGF mRNA was remarkably down-regulated by UVB at 4 hrs. These results suggest that NGF could act as a cytokine at the skin level and participate in the mechanisms associated with inflammatory dermatoses, such as psoriasis.

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ACTIVITIES OF ZINC AND TITANIUM OXIDES ON UVA/UVB-INDUCED INTERLEUKIN-6 PRODUCTION BY EPIDERMAL CELL LINES.

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Following UV irradiation, human keratinocytes produce and release numerous immunomodulatory cytokines, notably interleukin-6 (IL-6) which features prominently in the inflammatory reaction.

In the present study, we investigated IL-6 production by SVK14 keratinocytes after UVA and UVB irradiation, and it's modulation by zinc oxide (ZnO) and titanium dioxide (TiO2), two compounds frequently incorporated in commercially developed mineral sunscreens.

Cells were exposed for 24 hours to increasing subtoxic concentrations of ZnO or TiO_2 (6-25μM), then irradiated with UVA (2-4 J/cm^2) or UVB (5mJ/cm^2) and maintained in culture for 24 hours. IL-6 concentrations in the cell supernatants were measured by enzymatic immunoassay (EIA).

UVA at intensities of 2 and 4 J/cm^2 caused increases in the stimulation of IL-6 production of 47% and 92% respectively. These increases were significantly reduced by ZnO (10-30% reduction), with TiO_2 showing weaker inhibition. With regard to UVB irradiation of 5mJ/cm^2, IL-6 production increased 43%, being significantly inhibited by 6μM ZnO (45%), while TiO_2 had less inhibitory activity. These results highlight the interesting anti-inflammatory capacity of zinc oxide, and justify it's incorporation into sunscreen formulations.

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INVOLVEMENT OF FERROUS/FERRIC IRON IN THE UVB MEDIATED INDUCTION OF INTERSTITIAL COLLAGENASE (MMP-1) mRNA. Peter Brenneisen (1), Karlis Briviba (2), Meinhard Wlaschek (1), Jutta Wenk (1), Helmut Sies (2), Karin Scharffetter-Kochanek (1); Department of Dermatology, University to Cologne (1); Department of Physiological Chemistry I, University Düsseldorf (2), Germany.

Stratospheric ozone depletion is accompanied by an increase in the intensity of the UVB irradiation on the earth and increase of cellular reactice oxygen species (ROS). To better define the involvement of distinct ROS in the up-regulation of matrix-degrading metalloproteinases (MMPs) responsible for the connective tissue degradation in photoaging and tumor invasion, fibroblast monolayer cultures were subjected to defined doses of the total UVB spectrum (280-320 nm). These experiments were performed in the absence or presence of non-toxic concentrations of inhibitors for ROS detoxifying enzymes and for the Fenton reaction leading to an intracellular increase of distinct ROS. Furthermore, lipid peroxidation was inhibited and hydroxyl radicals were scavenged. A time- and dose-dependent increase in the MMP-1 mRNA levels with a maximally 6-fold induction at 24h post-irradiation was observed. The inhibitor of MMPs, TIMP-1, increased only marginally. The inhibition of detoxifying enzymes and of the Fenton reaction showed that iron chelators were able to inhibit the UVB-mediated induction of MMP-1 mRNA levels by 50-85% compared to the UVB irradiated controls. Furthermore, scavengers for hydroxyl radicals inhibited the MMP-1 mRNA induction by 70%. Inhibition of lipid peroxidation by a vitamin E derivative resulted in the reduction of MMP-1 mRNA levels by 55% compared to the control cells. These results point to the importance of ferrous/ferric iron in catalyzing hydroxyl radical and lipid peroxide formation upon UVB irradiation finally leading to the induction of MMPs. Taken together, this work has outlined preventive strategies which may stimulate further development of protective agents for photoaging and tumor progression.

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INDUCTION OF MATRIX-METALLOPROTEINASES IN RESPONSE TO REACTIVE OXYGEN SPECIES IN MANGANESE SUPEROXIDE DISMUTASE OVEREXPRESSING CELLS. Jutta Wenk (1), Peter Brenneisen (1), Meinhard Wlaschek (1), Karlis Briviba (2), and Karin Scharffetter-Kochanek (1), Dept. of Dermatology, Univ. of Cologne, Germany (1), Dept. of Physiological Chemistry I, Univ. of Düsseldorf, Germany (2).

Reactive oxygen species (ROS) are generated by ultraviolet irradiation (UV) in the skin and have been implicated in the pathogenesis of skin cancer, certain photodermatoses, and photoaging. To better define the role of distinct ROS in cytotoxicity and in up-regulation of matrix-metalloproteinases, we transfected the human dermal fibroblast cell line 1306 with an eukaryotic expression vector containing the human manganese superoxide dismutase (MnSOD) cDNA. Overexpression led to an increase in MnSOD activity by 4.8 fold and an increase in the spontaneous release of hydrogen peroxide (H_2O_2) by 60%. To further enhance the intracellular concentration of H_2O_2, MnSOD overexpressing fibroblasts have been subjected to UVA (H_2O_2↑) or paraquat treatment ($O_2^{\cdot-}$↑). After UVA irradiation (100 to 1500 kJ/m^2), no alteration in the viability of MnSOD overexpressing cells could be detected. In contrast, increased MnSOD activity in overexpressing cells, when treated with paraquat (50 to 750 μM), protected them against paraquat generated $O_2^{\cdot-}$ cytotoxicity. Paraquat exposure of MnSOD overexpressing cells led to a substantial increase in H_2O_2 release, suggesting that dismutation of $O_2^{\cdot-}$ to H_2O_2 reflects the cytoprotective mechanism. Interestingly, both UVA and paraquat treatment resulted in a significant increase in specific MMP-1 mRNA in MnSOD overexpressing cells. A similar increase in MMP-1 mRNA was also seen when the intracellular H_2O_2 concentration was increased by inhibition of H_2O_2 detoxifying glutathione peroxidase, catalase, and the $O_2^{\cdot-}$ consuming Fenton reaction. We show that enhanced MnSOD activity protects cells from $O_2^{\cdot-}$ mediated cytotoxicity, however, simultaneously, by imbalanced H_2O_2 overproduction and detoxification, induces MMP-1 and most likely tissue degradation in photoaging, tumor invasion and related processes.

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TNF-α AND IL-8 ARE UPREGULATED IN THE EPIDERMIS OF NORMAL HUMAN SKIN AFTER UVB EXPOSURE, CORRELATING WITH NEUTROPHIL ACCUMULATION AND E-SELECTIN EXPRESSION.

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The in vivo response to ultraviolet B (UVB) radiation in the skin, is characterised accumulation of both mononuclear and polymorphonuclear cells within the dermis and an induction of vascular endothelial adhesion molecules. Epidermal production of cytokines, (IL-8 and TNF-α) has been strongly implicated in the development of UVB-induced inflammation. In the present study, we examined the time course of IL-8 and TNF-α mRNA and protein expression in the epidermis over a 24 hour period after in vivo UVB irradiation. Also the induction of adhesion molecule expression and the accumulation of neutrophils within the dermis was followed. We found constitutive expression of both cytokines (mRNA and protein) in the epidermis of unirradiated skin. IL-8 was rapidly upregulated post-irradiation reaching a maximum between 8 and 24 hours. Similarly, TNF-α mRNA upregulation was evident by 4 hours post-UVB, reaching a maximum by 24 hours. However, no change in TNF-α protein expression was observable until 24 hours. E-selectin expression, which was absent from control samples, was increased from 4 hours onwards and also reached a maximum at 24 hours, coinciding with peak neutrophil accumulation. A strong correlation ($r = 0.96$) was found between number of E-selectin positive vessels and numbers of infiltrating neutrophils at this time point. Moreover, since E-selectin expression was increased before any apparent increase in TNF-α protein expression (4 hours), it is likely that this early increase is a direct effect of UVB on the vasculature rather than an indirect effect of TNF-α itself. No significant alteration in ICAM-1 or VCAM-1 expression was observed. The current data indicate a strong inter-relationship between UVB-induced epidermal cytokine expression and the subsequent inflammatory response in the skin.

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SOLAR SIMULATED RADIATION (SSR)-INDUCED CYTOKINE RELEASE AND MODULATION OF ANTIGEN PRESENTING CELL FUNCTION IN HUMAN SKIN *IN VIVO*. RM Barr, WL Tsang, SL Walker, GI Harrison, P Ettehadi, J Nagel, JLM Hawk, MWGreaves, AR Young. St John's Institute of Dermatology, UMDS, London, UK.

The kinetics of SSR-induced cytokine release in human skin *in vivo* and their relationship to cutaneous immunosuppression are not well defined. We measured the dose-response and time course of IL-1α, IL-1β, IL-10 & TNFα release and suppression of alloantigen presentation in SSR-irradiated human skin of types I/II. Suction blister epidermal tops and exudates were collected at either 15h after 0, 0.5,1,2 & 3 minimal erythema doses (MED) or at 4,8,15,24,48 & 72h after 3MED SSR. Cytokines were analysed by ELISA and alloantigen presentation determined *ex-vivo* by the mixed epidermal cell lymphocyte response (MECLR) assay.

TNFα was raised at 4h (59 to 282 pg/ml, unirradiated *v* 3MED, $p<0.001$, $n=16$), reached a maximum at 15h (80 to 688 pg/ml, $p<0.001$, $n=13$) before declining at 24h (62 to 212 pg/ml, $p=0.014$, $n=9$) to control levels by 48h. Increased TNFα at 15h was dose-dependent ($p<0.001$, $n=7$). SSR induced a small increase in IL-10 at 15h (28 to 50 pg/ml, $p=0.008$, $n=12$) but dose-dependency was not shown ($p>0.05$, $n=6$). IL-1β showed a substantial, dose-dependent increase at 15h (7 to 48 pg/ml, $p<0.001$, $n=11$), then fell but remaining above control until 72h. IL-1α showed a small increase at 15h (239 to 346 pg/ml, $p<0.05$, $n=17$) that was maintained until 72h. The MECLR was suppressed ($p<0.001$) by 70% between 4 and 15h after 3MED of SSR but returning to control levels at 24h.

These data show an early increase in TNFα release in SSR-irradiated human skin *in vivo*, preceding release of other cytokines, and concomitant with suppression of alloantigen presentation. In contrast, increases in IL-10 were small relative to the change in TNFα.

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ISOLATION AND CLONING OF cDNAs THAT ARE DIFFERENTIALLY EXPRESSED BETWEEN UV-RADIATED AND NON-RADIATED KERATINOCYTES: USING A DIFFERENTIAL DISPLAY TECHNIQUE.

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The differential display technique was applied to compare mRNAs from UVB-radiated and non-radiated human normal Keratinocytes (HNK) and a transformed human keratinocyte cell line (HaCaT). The cells were irradiated with UVB, 60 and 100 mJ/cm^2 respectively. After a culture period of 30 minutes and 4 hours, mRNA was extracted and amplified by reverse transcriptase-polymerase chain reaction (DDRT-PCR) using one-base anchored oligo-dT and arbitrary primers. Polyacrylamid gel elektrophoresis revealed several differentially expressed genes in untreated and UVB irradiated cells. cDNA fragments resulting from differentially expressed mRNAs were eluted from gel, reamplified and used as probes for Northern blot analysis to evaluate UV-associated gene expression. Northern Blot analysis revealed a dose dependent regulation of mRNA derived from UV-treated cells. cDNA showing significant changes by Northern blot analysis were subcloned using TA cloning kit and will be further analysed by sequencing. This data shows that DDRT-PCR is a powerful method for elucidating UV-regulated gene expressions. Identification of these genes by sequence analysis may lead to further insight into the role of specific genes in the UV- response in human keratinocytes.

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OVEREXPRESSION OF THE SMALL HEAT SHOCK PROTEIN, HSP27, CONFERS RESISTANCE TO HYPERTHERMIA BUT NOT TO OXIDATIVE STRESS AND UV-INDUCED CYTOTOXICITY IN A STABLY TRANSFECTED EPIDERMAL CELL LINE.

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The 27-kD heat shock protein (HSP27) is a member of the small heat shock protein (HSP) family. HSP27 may play a role in the regulation of cell growth and differentiation as well as in the protection of cells from stress induced cell damage. We have recently reported that HSP27 is expressed in human suprabasal keratinocytes and that its expression is associated with keratinocyte differentiation in vitro and in situ. This study was conducted to further investigate whether the expression of HSP27 is associated with increased resistance to the deleterious effects of UV radiation. A transfection vector carrying the human gene for HSP27 under the control of the HSP27 as well as the SV40 promotor (PSG2711, M.Jäättelä et al, EMBO J. 11:3507-3512, 1992) was introduced together with a neomycin-resistance gene into the epidermal carcinoma cell line A431. A mock-transfected clone was used as a control (A431-neo). Clones were screened for the expression of HSP27 using western blot and immunohistochemistry. A clone overexpressing HSP27 (A431-16) was used for further experiments. Cells were exposed to either UVA (Mutzhas, 315 nm - 390 nm), UVB (Mutzhas, 290 nm - 330 nm), heat (45°C for 1 h to 4 h), or H_2O_2 (400 μM - 1200 μM). Viability of treated cells was determined using a modified tetrazolium-based colorimetric assay. Overexpression of HSP27 induced increased resistance to hyperthermia but not to H_2O_2-mediated oxidative injury in A431-16 when compared to the mock transfected control cells. However, when cells were exposed to increasing amounts of UVA (10 J/cm^2 to 100 J/cm^2) and UVB (2 mJ/cm^2 to 64 mJ/cm^2) the percentage of surviving cells was identical for A431-16 and A431-neo. From these data we conclude that HSP27 is a mediator of thermotolerance but does not protect epidermal cells from UV-induced cell death.

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EXPRESSION OF THE 72-KD HEAT SHOCK PROTEIN IS INDUCED BY ULTRAVIOLET LIGHT A.

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The 72-kD heat shock protein is expressed in all cells and tissues upon exposure to elevated temperatures (heat shock). Hsp72 is functionally associated with a transient state of increased cellular resistance to further stress challenge. We have reported recently that epidermal keratinocytes express high levels of hsp72 without previous heat stress. It has been further demonstrated that heat treatment is able to induce increased resistance to the deleterious effects of UVB in human epidermal keratinocytes in vivo and in vitro. Hsp72 is a mediator of this protective effect. In this study we further investigated whether ultraviolet radiation is able to induce hsp72-expression and whether this induction is associated with increased resistance to UVB-induced cell death. The human fibrosarcoma cell line HT1080 was selected for these experiments because hsp72 is not detectable in these cells at normal culture conditions. Cells were either treated with UVA (Mutzhas, Germany, 315 nm - 390 nm) or with UVB (Mutzhas, 290 nm - 330 nm) and hsp72 was determined in whole cell extracts by immunoblotting using a monoclonal antibody (Amersham). Non radioactive band shift assays were employed for detection of heat shock factor (hsf) and northern blots using a cDNA probe for detection of hsp72 mRNA. Heat treated HT1080 were used as a positive control. At the protein level hsp72 could not be detected after treatment with UVB (4 mJ/cm^2 to 32 mJ/cm^2). In contrast, upon treatment with UVA (10 J/cm^2 to 80 J/cm^2) hsp72 was induced with a maximum at 40 J/cm^2 8 h to 12 h after Uv exposure. Hsf and hsp72 mRNA were detected 30 min and 2 h after induction with UVA, respectively. However, densitometry of western blots demonstrated a 10-fold lower induction of hsp72 by UVA compared to heat shock. Furthermore, UVA induced expression of hsp72 was not accompanied by an increased resistance of HT1080 to cell death induced by subsequent exposure to UVB. These data for the first time demonstrate UVA-induced expression of hsp72. UVA-induced hsp72 may be involved in the cellular protection from environmental stress.

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THE EFFECT OF TRETINOIN ON THE DAMAGING EFFECTS OF ULTRAVIOLET RADIATION.

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Ultraviolet radiation (UVR) is a major factor in the aetiology of skin cancer. UVR induced damage to the skin can be measured by inhibition of Langerhans cell (LC) alloantigen presentation using the mixed epidermal/lymphocyte reaction (MELR) indicating reduced immunosurveillance and by upregulation of which indicates damage to cellular DNA.

The effects of tretinoin on UV induced skin damage are controversial with studies suggesting a protective effect and others a potentiating effect. Using an explant system we have investigated the effect of 0.05% tretinoin in an emollient cream base on UVR induced skin damage. Fresh skin from cosmetic surgical procedures was cut into 6cm^2. 24µl of 0.05% tretinoin, moisturiser lotion or phosphate buffered saline was applied to the surface of the explant and gently rubbed into the surface in a standardised fashion and incubated in a humidified environment for 2 hours at room temperature or 37°C. The explants were irradiated using an FS20 sunlamp with 18.5-740mJ/cm^2 UVB. The explants were decontaminated and either processed for an MELR or incubated at 37°C for 20-24 hours after which p53 was identified by Western blotting using the mouse antihuman p53 antibody, DO1.

Results demonstrated a UV dose dependant reduction in LC function and upregulation of p53 in saline and moisturiser treated skin. Both inhibition of LC function and upregulation of p53 were significantly reduced by the prior application of 0.05% tretinoin.

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THE EFFECTS OF CALCIPOTRIOL AND CLOBETASOL-17-PROPIONATE ON ULTRAVIOLET B IRRADIATED HUMAN SKIN: AN IMMUNOHISTOCHEMICAL STUDY

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It was shown previously that the effect of an intermediate dose ultraviolet B (UVB) on healthy skin mimics a newly developing psoriatic lesion. The aim of the present study is to compare and contrast the effects of the vitamin D_3 analogue calcipotriol, clobetasol-17-propionate (clobetasol) and the ointment base of calcipotriol on normal human skin challenged with an intermediate dose of UVB. Immunohistochemical markers for epidermal growth (Ki-67), keratinisation (cytokeratin 16, involucrin, transglutaminase) and inflammation (CD2, CD1a, elastase) were studied on frozen histological sections and an assessment on the atrophogenicity of the ointments was conducted.

Clobetasol proved to inhibit UVB induced epidermal hyperproliferation, keratin 16 induction and accumulation of T lymphocytes and CD1a positive cells and induced epidermal thinning. No effect on keratinisation was seen. In contrast, calcipotriol only reduced the number of transglutaminase positive cell layers and increased the thickness of the epidermis.

The present study reconfirms the 'broad-spectrum' effect of clobetasol on various aspects of UVB challenged skin. In contrast, calcipotriol barely modulated UVB induced changes. These findings might be considered to be in line with the observation that calcipotriol is relatively less effective in the approach of newly developing psoriatic lesions.

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The Photoprotective Effect Of Ascorbic Acid, Acetylsalicylic Acid, And Indometacin evaluated by the Photo Hen´s Egg Test.

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The photo hen´s egg test (PHET) as a model for phototoxicity is more advanced than the widely used cultures of yeasts, bacteria or cell cultures of various origin. The hen´s egg test was originally introduced by toxicologists as a screening model for mucocutaneous toxicity as an alternative to the rabbit´s eye test (Draize test). The aim of this study was to test the supposed photoprotective effect of ascorbic acid (AA), acetylsalicylic acid (ASA), and indometacin (IN) employing the PHET. Therefore, in three independent experiments two groups (n=12 each) of embryo´s yolk sac blood vessel systems (YS) were exposed to 60 mJ/ cm^2 UVB (290-320 nm, Philips TL 12W/09) to induce severe phototoxic damage. Prior to the UVB irradiation, one of these groups was additionally exposed to one of the test substances in a nontoxic concentration (AA 10^{-2}, ASA 10^{-2}, IN 10^{-3} molar in 500µl PSS) and the other two groups were exposed to 500 µl PSS or to one of the test substances alone. For a period of 24 hours, the morphological parameters membrane discoloration (MD) and hemorrhage (HR) were monitored and graded. Additionally the lethality was observed. As expected, a pronounced phototoxic damage of the YS was observed in the PSS/UVB group. The UVB damage (severe MD 83%, severe HR 17%, lethality 58%) was reduced by AA (severe MD 42%, severe HR 0%, lethality 8%). Also ASA (severe MD 58%, severe HR 0%, lethality 33%) could reduce the UVB damage (severe MD 100%, severe HR 25%, lethality 83%). In our test concentration IN (severe MD 92%, severe HR 42%, lethality 67%) was not able to reduce the UVB damage (severe MD 67%, severe HR 0%, lethality 67%). In the controls, none of the embryos died, and only slight morphological changes were observed. Thus, AA and ASA were able to reduce significantly UVB induced damage of the YS. Although IN is known to decrease prostaglandin synthesis, no photoprotective effect was detected in the PHET.

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COMPARISON OF ACUTE AND CHRONIC ULTRAVIOLET (UV) B INDUCED INFLAMMATION. D. Bevan, F.M. Cunningham[1], J.G. Meingassner & H.D. Moore, Sandoz Reasearch Institute, Dept. of General Dermatology, Vienna, Austria and [1]The Royal Veterinary College, Hawkshead Campus, North Mymms, Hertfordshire, U.K.

We have recently described similarities between the histological appearance of porcine skin irradiated daily for 4 weeks with a sub-erythemal dose of UVB and the chronic inflammatory changes seen in certain skin conditions in man [Bevan *et al.*, Skin Pharmacol.;1996; 9:143]. This study compares changes seen after a single UVB exposure (acute response) to those observed after repeated irradiation (chronic response). The minimal erythemal dose (MED) was found for each domestic pig (4 per group) and fresh sites were then irradiated once with 0.5 or 2 MED of UVB emitted from a bank of 6 lamps (Philips, TL20W/12). Skin samples were taken after 8, 24, 48 and 72 h, fixed, sectioned, Giemsa stained for histological and epidermal thickness evaluation and stained with anti-PCNA for measurement of keratinocyte proliferation. The dermal mononuclear cell (MNC) infiltrate was increased at each time point after a single 0.5 MED exposure but was only significant after 8 and 24 h ($p<0.05$). Neutrophil and mast cell (MC) numbers remained unchanged throughout the study. Increased PCNA immunoreactivity was seen at 24 h without significant increase in epidermal thickness. Similar changes in MC and MNC numbers were seen following a single 2 MED exposure. In addition, neutrophil numbers were significantly increased at each time point. A significant increase in PCNA immunoreactivity was detected after 24 and 48 h and in contrast to 0.5 MED irradiated sites, this was accompanied by a transient increase in epidermal thickness, returning to near baseline levels after 72 h. Extensive linear parakeratosis, acanthosis and hyperkeratosis were evident 72 h after both 0.5 and 2 MED irradiations. These results demonstrate that, although a single sub-erythemal UVB exposure can cause MNC accumulation and keratinocyte proliferation in normal porcine skin, repeated exposure of this dose is required to induce a change in epidermal thickness. Increasing the dose to 2 MED was sufficient to cause an increase in epidermal thickness however this was not sustained. Moreover, the neutrophil infiltrate present in these sites was indicative of an acute response. These data illustrate the similarities between the response of porcine and human skin to a single dose of UVB and further demonstrate the suitability of repeated low dose exposure of porcine skin as a model for the chronic inflammatory changes in certain skin conditions in man.

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ANTAGONIC EFFECTS OF INFRARED AND ULTRAVIOLET A RADIATIONS ON NORMAL HUMAN DERMAL FIBROBLASTS. S Menezes, C Lebreton, L Dubertret, B Coulomb. Inserm 312, Lab. Dermatology, St-Louis Hospital, Paris - France.

This work was designed to study the influence of an IR (far-red) pre-irradiation on the UV-A induced cytotoxic effects. Normal human skin fibroblasts were cultured in phenol-red-free EMEM medium, without antibiotics. Confluent monolayers were washed with Hank's salt solution, then irradiated with IR and/or UV-A. IR irradiation was performed with an IR GE 27, 250 W lamp, at a distance of 42 cm, for 15, 30 or 45 minutes. During this irradiation, the temperature of the solution was maintained at 20°C by a water-cooling system. For UV-A (365 nm) irradiation, doses of 25 and 35 J/m^2 were used, at 37°C. For double irradiations, UV-A followed IR immediately. Membrane damages were evaluated by dosing the TBARS (thiobarbituric acid reacting substances). Cell viability was measured by counting cells 24 hours after irradiations. Heat-shock proteins induction was detected using mouse monoclonal antibody specific for HSP 72 and fluorescent isothiocyanate (FITC)-labeled goat antimouse IgG as a second antibody.

- Few hours after IR irradiation, the number of mitotic cells was augmented; - IR pre-irradiation strongly inhibited the UV-A cytotoxic effects; - When cellular mitosis was blocked by Ara-C (1µg/ml), IR inhibition of UV-A cytotoxic effects was still present, indicating that the protection was not due only to the stimulation of mitosis; - IR did not affect the UV-A strongly induced TBARS augmentation, indicating that the protection is not related to membrane lipid peroxidation; - IR irradiation did not induce HSP 72 immediately or after 30, 60 or 120 minutes of post-irradiation incubation either in Hank's solution or complete medium, while cells treated fo 15 minutes at 45°C exhibited strong induction of this protein under all those conditions.

In conclusion, IR (far-red) irradiation, without increasing temperature nor HSP induction, protects fibroblasts from UV-A cytotoxic effects. This work shows also that it is important to consider the interactions between different wavelengths when studying cell behavior in response to solar irradiation.

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ICG-MEDIATED PHOTOTHERAPY FOR THE TREATMENT OF KAPOSI'S SARCOMA
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Necrosis of tumors following Photodynamic Therapy (PDT) is due to the destruction of the tumor microvasculature and induction of tumor ischemia (vascular targeting). However, an effective therapy is restricted to superficial tumors (< 4mm) so far. To treat larger tumors photosensitizers are needed which absorb in the near infrared region of the visible spectrum allowing deeper tissue penetration of light. Indocyanine green (ICG) with an absorption max. at 805 nm in vivo is a clinically approved dye and bound to plasmaproteins after i.v. injection. Thus ICG is confined to the intravascular space allowing targeting of the tumor microvasculature directly after injection. Due to these intriguing characteristics phototherapy mediated by ICG was used for the first time to treat AIDS-associated Kaposi's sarcoma (KS), as model of a highly vascularized tumor.

A total of 57 mainly nodular lesions (Ø 0.4 - 2.1 cm) in 3 patients with KS were treated. After injection of ICG (2.0 - 5.0 mg/kg b.w.; ICG-Pulsion, Munich, Germany), in two doses 30 min apart, lesions were irradiated immediately up to 40 min following the second injection with a total light dose of 100 J/cm^2 and an intensity of 0.5 - 5 W/cm^2 using a diode laser (805 nm). Surface skin temperature was measured by a thermocouple before and directly after irradiation. Biopsies were taken 24 h and 4 weeks after therapy.

Best results (100% complete remission) were obtained using a concentration of 5.0 mg/kg b.w. administered in two doses using a total light dose of 100 J/cm^2 (3-5 W/cm^2). Biopsies 24 h after irradiation showed total necrosis of KS. Biopsies taken 4 weeks after therapy revealed only superficial scar formation. Clinically lesions on the trunk showed cosmetically excellent results, whereas lesions on the lower extremities healed with slight hyperpigmentation. Mean temperature increase of skin after irradiation was 10.4°C indicating a photothermal effect.

This report shows that AIDS-associated KS can be effectively destroyed by ICG-mediated phototherapy without systemic side effects. A large number of lesions can be treated in one session with excellent cosmetic results. However, the major advantage as compared to PDT with Photofrin® (630 nm) is the clinical approval for ICG in most countries and the deeper tissue penetration due to absorption in the near infrared (805 nm).

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HUMAN SKIN MITOCHONDRIAL DNA DELETIONS ASSOCIATED WITH ULTRAVIOLET LIGHT EXPOSURE AND AGEING SKIN. Mark A. Birch-Machin and Jonathan L. Rees. Department of Dermatology, University of Newcastle upon Tyne, United Kingdom.

Mitochondrial DNA (mtDNA) mutations have recently been proposed as important contributors to ageing and neurodegenerative diseases. Mitochondria are intracellular organelles which are key elements in aerobic metabolism from which a great deal of reactive oxygen species are continuously generated. MtDNA is a naked double-standed circular molecule that is continuously exposed to the matrix which contains great amounts of reactive oxygen species. Among the mtDNA mutations reported so far, deletions have received more attention than point mutations in the contribution to human ageing. The most common mtDNA deletion identified in various tissues of elderly subjects is the 4977 bp deletion, the so-called common deletion. Most of the age-associated changes in skin, including the increased incidence of skin cancer and the impaired wounding responses, reflect long term damage from ultraviolet radiation rather than intrinsic ageing. Skin therefore represents one of the most suitable human tissues for studying the interaction between intrinsic and extrinsic ageing processes such as UV-induced DNA damage. Due to the large number of mutations that may occur in mtDNA, we have simplified our approach by measuring the incidence of the common deletion and used it as an index of overall damage of skin mtDNA. We present data from our investigations of the incidence of the common mtDNA deletion in normal and tumour skin biopsies taken from different UV-exposed body sites and from differently aged subjects. Using a phosphoimager technique we have quantified the proportion of delete mtDNA species (ranging from 1-50%) compared to wild type.

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COLLAGEN TYPE VI IN PHOTOAGED SKIN
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Photoaged skin differs substantially from intrinsically aged, photoprotected skin in exhibiting wrinkles and actinic lentigines. Little is known about the histological changes underlying wrinkle formation although changes in the dermal matrix include increased deposition of elastotic material in the papillary dermis, and decreases in collagens I, III and VII.

In this study, we investigated the distribution of collagen VI, an abundant and important structural component of dermal matrix. We have examined collagen VI in photoaged vs photoprotected skin using immunohistochemistry and confocal microscopy. Subjects adjudged to have clinically moderate to severe photoageing (n=10) and mild photoageing (n=3) were recruited to the study. Punch biopsies (4mm diameter) were taken from extensor forearm (photoaged site) and from photoprotected hip and upper inner arm (as an anatomical site control). Seven µm frozen sections were stained with polyclonal antibody (5508) raised against intact collagen type VI microfibrils, omission of the primary antibody constituting the negative control. Visualisation was via an anti-rabbit Cy3-conjugated secondary antibody. Sections were randomised, blinded and assessed on a 5 point, semi-quantitative scale (0=no staining, 4=maximal staining; three high power fields per slide; three slides per biopsy area).

In photoprotected skin, collagen type VI was concentrated in the papillary dermis immediately below the dermal-epidermal junction and in the deeper dermis around blood vessels, hair follicles and glandular structures (hip=2.29 ±0.64; upper inner arm = 2.03±0.65). In photoaged skin, there was no significant alteration in the pattern of collagen type VI deposition (forearm = 1.97±0.54).

This study suggested that collagen type VI is a constituent component of human dermis and its distribution and abundance is not substantially altered following chronic UV-irradiation. Thus, collagen VI, unlike other collagens so far studied, appears not to contribute to the gross structural changes observed in photoageing.

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CELLULAR MECHANISM OF AGE-RELATED EARLY DECLINE OF DELAYED-TYPE HYPERSENSITIVITY RESPONSE IN SAMP1 MICE. Eiko Toichi[1,3], Masamichi Hosono[2], Masanori Hosokawa[1], Sadao Imamura[3], [1]Depts. of Senes. Biol. and [2]Immunol., Chest Dis. Res. Inst. and [3]Dept. of Dermatol., Fac. of Med., Kyoto Univ., Kyoto, Japan.

Delayed-type hypersensitivity (DTH) response to sheep red blood cells in SAM (Senescence Accelerated Mouse) P1 mice declined after 7 months of age and reached the base level by 10 months, when that in control mice was still high. The transfer of spleen cells from aged mice, sensitized for DTH response, into the footpads of naive mice with the antigen (Ag) , evoked strong DTH response, demonstrating the existence of DTH-mediating T (T_{DTH}) cells in the spleen of aged SAMP1 mice. On the other hand, the local transfer of nylon-wool-purified T cells from young donors showed DTH response in young recipients, but not in aged recipients. Then, to find out whether some factors except T_{DTH} cells are insufficient at the Ag-challenged site in the aged, we injected spleen cells from naive young donors, which had been deprived of T cells, into the footpads of sensitized aged recipients together with challenge Ag. These aged recipients expressed sufficient DTH responses. These data strongly suggest that T_{DTH} cells are relatively resistant to the effects of aging and that the recruitment of cells except T_{DTH} cells, which are involved in the effector phase of DTH response, is impaired in "aged" SAMP1 mice.

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CROSS-LINKING OF THE DERMO-EPIDERMAL JUNCTION IN REGENERATING SKIN: ANCHORING FIBRILS ARE A TARGET FOR TISSUE TRANSLUTAMINASE

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Since transglutaminases (TG) create covalent γ-glutamyl-ε-lysine cross-links between extracellular matrix proteins they are prime candidates for stabilizing tissue during wound healing. We studied the temporo-spatial expression of TG activity in skin regenerating from cultured epithelial autografts in severely burned children by the specific incorporation of monodansyl-cadaverine into cryostat sections from skin biopsies obtained between 5 days to 17 mo post grafting. The dansyl label was subsequently immunolocalized in the epidermis, dermal connective tissue and along the basement membrane. Incubation of normal and regenerating skin with purified tissue TG confirmed the dermo-epidermal junction and the papillary dermis as targets for this enzyme and revealed that in regenerating skin transamidation of the basement membrane zone was completed only 4 - 5 mo post grafting. This time point coincided with the clinical stabilization of the autografts. Immunoelectron microscopy revealed three distinct regions on the central portion of anchoring fibrils positive for monodansylcadaverine which were not labeled during the initial phase of de novo formation of anchoring fibrils. Biochemically, we identified the triplehelical region of collagen VII as a poor, but the carboxyterminal NC-2 domain as good potential glutaminyl substrate for tissue TG. Thus, tissue TG appears to play an important role not only as a crosslinker of the dermo-epidermal junction.

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GROWTH FACTOR GENE THERAPY: PRODUCTION OF WOUND HEALING GROWTH FACTORS BY GENETICALLY MODIFIED SKIN GRAFTS

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The transplantation of cells combined with biomaterials is one strategy for organ replacement or tissue repair for a variety of therapeutic needs, including skin replacement after extensive skin loss. The present study seeks to determine if genetic modification of the cells can be used to enhance the performance of a cell-biomaterial composite used as a bioartificial skin. Retroviral-mediated gene transfer was used to introduce into diploid human keratinocytes the gene encoding human platelet-derived growth factor A (PDGF-A). Gene modified keratinocytes were seeded onto human de-epidermized acellular dermis (DED) *in vitro* and secreted PDGF-A at a rate of 90ng/graft (1.5x1.5 mm^2)/24 hours. Composite grafts with modified and unmodified control cells were transplanted to excisional wounds on the back of athymic mice. Seven days after grafting modified keratinocytes underwent terminal differentiation and generated a stratified epidermis comparable to unmodified cells. In contrast, the DED subjacent to PDGF-A secreting keratinocytes had significantly increased numbers of fibroblasts, mononuclear cells, and blood vessels, when compared to control grafts at day 7. The fibrovascular ingrowth into the DED induced the replacement of the human dermal template as indicated by an increase of mouse collagen I. PDGF-A expression reduced graft contraction by 50 % when compared to grafts of unmodified cells at day 28. This study suggests that genetic modification of the cells in a cell-biomaterial composite is a means to enhance the function of bioartificial skin.

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COLLAGENASE-3 IS EXPRESSED BY STROMAL CELLS IN CHRONIC ULCERS BUT NOT IN NORMALLY HEALING WOUNDS.

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Collagenase-3 (MMP-13) is a novel metalloproteinase that differs from interstitial collagenase (MMP-1) by having a broader substrate specificity. As shown in our earlier work, MMP-1 plays an important role in wound healing. This study was carried out to compare the patterns of expression of these two related metalloproteinases in chronic ulcers and in normally healing wounds.

In situ hybridization using ^{35}S-labelled cRNA probes for MMP-1 and -13 was performed on formalin-fixed paraffin-embedded samples from nine chronic venous ulcers (6 mo - 9 yr old) and twelwe normally healing wounds on the thigh, representing biopsies 2 - 7 d post wounding.

MMP-13 was detected in all specimens of chronic ulcers in spindle, fibroblast-like and plump, macrophage-like cells, whereas all normally healing wounds were devoid of signal. MMP-1 was expressed in both acute and chronic wounds in the basal keratinocytes bordering the ulcer, while MMP-13 mRNA was never detected in the epidermis. The spatial distribution of MMP-13 mRNA was different from that of MMP-1, extending deeper in the dermis.

Our results suggest that MMP-13 takes part in connective tissue turnover occuring during wound healing and is in vivo differently regulated from MMP-1.

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RECOMBINANT HUMAN TISSUE INHIBITOR OF METALLOPROTEINASE-2 (rh-TIMP-2) ACCELERATED WOUND HEALING.

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We obtained excellent effects of rh-TIMP-2 on wound healing in healthy rats, aged mice and diabetic mice. We histopathologically examined the processes of wound healing by rh-TIMP-2 as compared to control and investigated the *in vitro* effects of rh-TIMP-2 on the growth of human normal epidermal keratinocyte (NHEK) and human dermal fibroblast (HDF). rh-TIMP-2 (20 μ g daily) was applied to full thickness, 8 mm round incisional wound on the dorsal surface of female diabetic KK-Ay mice, and the wound was histopathologically examined at the course of healing. We histopathologically confirmed that wound closure was faster and the epidermis was thicker when wound was treated with rh-TIMP-2. In culture, the growth of HDF was stimulated in dose dependent manner above 3 μ g/ml and that of NHKE was suppressed with above 1 μ g/ml. These results suggest that rh-TIMP-2 stimulates growth of cells and enhances the migration of epidermal keratinocyte resulting in the enhancement of wound healing.

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LAMININ-5 IN MIGRATING KERATINOCYTES DURING THE RE-EPITHELIZATION OF HUMAN SKIN.

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The aim of this study was to investigate the distribution and the synthesis of laminin-5 (a component of anchoring filaments) during the controlled re-epithelization of human skin.

The suction blisters were induced on the abdominal skin of twelve volunteers, and the excisional skin samples were taken at 0, 2, 4, and 9 days after blister induction. The samples were studied with polyclonal antibody to the laminin-5 and *in situ* hybridization was done by the laminin-5 γ2 chain mRNA.

In fresh blisters, laminin-5 immunoreactivity was found variable both in the dermal side of blister cavity and detached epidermis. In regenerating epidermis laminin-5 staining was found cytoplasmically in keratinocytes, about 20-50 cells from the leading edge of the new epidermis. Thereafter the staining was linear in basement membrane region and more intensive under the regenerated epidermis than in normal skin. In nine days old blister, the blister scar was completely covered by a new acanthotic epidermis, and laminin-5 was expressed as a continuous line in basement membrane region. *In situ* hybridization revealed abundant number of signals for laminin-5 γ2 chain mRNA in 15-30 cells from the leading edge of the new epidermis.

The results indicate that during re-epithelization of wound with intact basement membrane, there is a need for transient synthesis and deposition of laminin-5 to facilitate the adhesion and migration of keratinocytes.

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INFLUENCE OF THE INTEGRITY OF THE SKIN PERMEABILITY BARRIER ON EPIDERMAL PROLIFERATION AND DIFFERENTIATION.

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Several previous studies have demonstrated that perturbation of the permeability barrier induced epidermal hyperproliferation. Our aim was to investigate whether there is a correlation between the degree of damage to the barrier, the extent of proliferation, and changes in terminal differentiation of the keratinocytes.

For looking for dose dependent differences 6 volunteers were treated with different concentrations of sodium lauryl sulfate (SLS). Measurements of transepidermal water loss (TEWL) were done before and after irritation. Punch biopsies were taken after 4 days. For investigation of interindividual differences in the degree of TEWL increase and epidermal reaction to the irritant a second experiment was performed in which 20 volunteers were treated only with a 0,5 % SLS solution. Measurements and biopsies were performed as described before. Histological sections were stained using 3 proliferation markers (PCNA, MIB 1, Ki-S1), antibodies against filaggrin, involucrin, and cytokeratines 10/11 and 6/16.

Irritation resulted in a SLS-dose-dependent increase of TEWL and proliferation. The individual different susceptibility to the irritant measured by the degree of TEWL increase after irritation with the same SLS concentration showed no correlation to the extent of proliferation. After irritation, involucrin and filaggrin were expressed stronger and also in deeper layers of the epidermis, whereas the expression of cytokeratines 6/16 increased slightly and cytokeratines 10/11 decreased dependent on the concentration of the irritant. Also in this experiment the degree of TEWL increase was not followed by a similar extent of differentiation change.

In summary we could demonstrate that proliferation and differentiation of the epidermal keratinocytes after damage to the skin permeability barrier depended more on the concentration of the directly cytotoxic acting irritant than on the degree of barrier perturbation.

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TAPE-STRIPPING OF HUMAN STRATUM CORNEUM YIELDS INCOMPLETE CELL LAYERS DUE TO CURVATURES IN THE SKIN

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Tape stripping of stratum corneum (SC) is widely used as a method to study the kinetics and depth of percutaneous absorption. The human epidermis, however, possesses macroscopic curvatures, which expand in a millimeter range parallel to the surface of the skin. These curvatures can cause severe problems when studying the superficial layers of the stratum corneum with the tape stripping technique. In a study to the penetration characteristics of a TiO_2 containing compound on tape-stripped human SC with X-Ray Micro Analysis (XRMA), we, unexpectedly, could trace titanium in the 4th strip, immediately after application of TiO_2. A light microscopical section of skin that was tape-stripped 30 times, clearly showed the non stripped skin in the furrows indicating persistent incomplete stripping. We have developed a mild replication technique, in which a monomeric form of a poly addition resin was spread over the skin and allowed to polymerize. Replicas obtained after repeatedly stripping were processed for the Scanning Electron Microscope (SEM). We validated the stripping method with XRMA in the mapping mode in the SEM, using the TiO_2 containing compound as a marker. This allowed the establishment of the stripping depth on basis of the presence or absence of titanium in the successively stripped areas from top to bottom of the curvatures. The results show that the curvatures are still present, even after 40 strips, and that after the 10th strip titanium is specifically located in the furrows. It is emphasized that results obtained by the stripping methods have to be seen in the perspective that one strip of the SC may possibly be derived from different layers depending on the position of the tape strip in relation to the slope of the curvature. This has led us to the conclusion that one has to be very careful with the interpretation on basis of tape stripping experiments when the penetration capacities of certain agents into the human stratum corneum are studied.

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TIME COURSE OF EPIDERMAL BARRIER RECOVERY AFTER ACETONE- OR TAPE-STRIPPING-INDUCED BARRIER-PERTURBATION IN ATOPIC ECZEMA AND NORMAL SKIN.

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Altered epidermal barrier function as determined by transepidermal water loss (TEWL) is a typical feature of patients with atopic eczema (AE). The purpose of this study was to assess the kinetics of epidermal regeneration after barrier-perturbation by acetone-treatment (removal of stratum corneum (SC) lipids) and tape-stripping (removal of the nonviable SC).

15 patients with AE and 12 nonatopic healthy normal controls were investigated. A skin area of 1.6 cm^2 on clinically normal skin of the forearm flexor side was treated by acetone or tape-stripping. After this treatment, TEWL-rates increased to 3.5-4.0-fold of the pretreatment value. TEWL-values were recorded directly after perturbation (t0), after 15 min. (t1), 3 h (t2), 6 h (t3), 24 h (t4), 48 h (t5), 72 h (t6) and 96 h (t7).

The rapidity of epidermal regeneration was faster after acetone treatment in the patient and the control group, there was no significant difference between the groups. However, after tape-stripping at points t2,t5 and t6 TEWL-values relative to t0 were significantly lower in atopic skin as compared with normal skin ($p<0.05$).

After barrier perturbation first a secretion of lamellar body lipids and secondly lipid synthesis (first cholesterol, later ceramides, which are decreased in AE) is stimulated. The faster regeneration of barrier function after tape-stripping in patients with AE may be a result from a persisting mild disturbance of barrier function in these patients. It may be speculated, that the repair mechanisms are permanently activated, and therefore barrier recovery is faster. However, a complete restoration of the epidermal barrier function is not be achieved, perhaps because of the decreased content of ceramides in atopic skin.

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MATURATION OF THE EPIDERMAL BARRIER IN AIR-EXPOSED KERATINOCYTE CULTURES: A TIME COURSE STUDY.

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Recently we could show that an air-exposed (A/E) keratinocyte culture reconstituted in chemically defined medium formed a structurally and biochemically normal permeability barrier. The purpose of the present study was to characterize the morphological sequence of in vitro barrier maturation. The A/E cultures were harvested from day 2 to 21 after air exposure. At each time point, the cultures were split for immunohistochemical (differentiation markers: KL1, CK 4, 6, 10, 13 , 16, involucrin, filaggrin, loricrin; proliferation markers: PCNA, MIB 1), and ultrastructural (OsO_4 and ruthenium tetroxide (RuO_4) post-staining) studies. Days 2,3 showed that normal structured lamellar bodies (LB) were already present in the cytoplasm but no secretion of LB-lipids was observed. Additionally, rounded keratohyalin granules (KH) instead of normal stellate KH were present. The corneocytes showed intracorneocytic lipid droplets. After 16-21d a normalization of differentiation (CK61, CK161, involucrin1), proliferation (PCNA1, MIB 11) and structure (stellate KH) was seen. Unfurling of secreted LB lipids and reorganization into mature lipid bilayers were observed. Additionally, there was a reduction of the intracorneocytic lipid droplets. This study showed that a structurally normal barrier was formed between day 16-21 in vitro. The barrier maturation process seems to parallel closely in vivo ontogenesis.

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TOPICAL TREATMENT BY PHORBOL ESTER PMA ± OCCLUSION IMPAIRS EPIDERMAL PERMEABILITY BARRIER

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Previously we have shown that disruption of the epidermal permeability barrier in mouse skin by acetone leads to an increase in epidermal DNA synthesis resulting in epidermal hyperplasia (J. Clin. Invest. 87, 1668-1673 (1991)). We also showed that occlusion by a latex wrap reduces the increase in DNA-synthesis and epidermal hyperplasia. We now asked, if vice versa, application of protein kinase c activator phorbol ester PMA, which is known to induce epidermal hyperplasia, results in disruption of the permeability barrier. We also asked if occlusion can reduce the increase in DNA synthesis and epidermal hyperplasia.

30µl PMA in isopropanol (0.25mM) was applied on one flank of hairless mouse skin. TEWL was recorded at different points of time 0-120 hrs. after treatment. In addition, biopsies were taken and histochemical (hematoxylin and eosin) and immunohistochemical studies (BrdU) were performed.

An application of PMA ± occlusion leads to a progressive increase in TEWL; at 48 hrs. a 3-fold increase in TEWL was obtained. Epidermal DNA synthesis was increased 2-3-fold and a 45% increase in thickness of the epidermis was obtained. The same increase in proliferation and epidermal thickness was also determined after occlusion.

These results show that induction of epidermal hyperproliferation by phorbol ester PMA leads to disruption of the permeability barrier. Occlusion does not reduce the increase in DNA synthesis and epidermal hyperproliferation induced by this drug.

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ASSESSMENT OF THE POTENTIAL IRRITANCY OF OLEIC ACID ON HUMAN SKIN: EVALUATION *IN VITRO* AND *IN VIVO*.

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As skin barrier modulating compounds, fatty acids are frequently used in formulations for transdermal or topical drug delivery. Therefore it is essential to minimize their potential of causing skin irritation. The aim of our study was to compare the *in vitro* human skin cell toxicity of the long chain unsaturated fatty acid, oleic acid, with its *in vivo* skin irritancy in humans.

Dose- and time-dependent effects of oleic acid in propylene glycol were evaluated in submerged human keratinocyte cultures, in reconstructed human epidermis (RE-DED), and in excised human skin specimens, using alterations in morphology and changes in interleukin-1α mRNA levels as endpoints. *In vitro* results were compared to responses of living human skin after topical application of oleic acid, using non-invasive bioengineering methods.

In submerged cultures cell toxicity was achieved at very low concentrations of oleic acid. No morphological alterations were observed following topical application on RE-DED or on excised skin. Modulation of stratum corneum thickness indicated a key role of the skin barrier in the control of oleic acid-induced toxicity. Although no epidermal damage was seen *in vivo*, measurable irritation was induced. In RE-DED and in excised skin IL-1α mRNA expression was increased when much higher concentrations were applied compared to the concentrations needed in submerged cultures.

The results suggest that, due to the rate-limiting transport of oleic acid across the skin barrier, skin irritation by the fatty acid occurs via a mechanism other than direct cytotoxic interaction with the living cell layers.

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Skin ceramide composition of atopic dermatitis patients.

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Patients with atopic dermatitis (AD) very often have dry skin associated to impaired barrier function and increased TEWL values both on normal and diseased skin. A few studies suggested that a reduced amount of total ceramides (especially of ceramide 1) deriving from epidermal keratinocytes may be responsible for functional abnormalities of the skin. In this study we analyzed the quantity of ceramides per unit mass of stratum corneum, cholesterol sulfate and free cholesterol of 47 AD patients and 20 age and sex matched healthy subjects . Stratum corneum sheets were removed from the forearm skin by stripping with cyanoacrylate resin and placed in hexane/ethanol to extract stratum corneum lipids. The stratum corneum was dispersed by solubilization of cyanoacrylate resin with dimethylformamide, and after membranc filtration, the weight of the stratum corneum mass was measured. The ceramides (CER), cholesterol free and cholesterol sulfate (CS) were analyzed by thin layer chromatography and quantified by videodensitometry. In AD patients, we observed a different distribution of ceramide classes and of cholesterol. Moreover, skin levels of ceramide 1 and 3 were significantly decreased (ceramide 1 was 5.5±2.4 % in AD and 4.2±1.8 in the normal population) and values of cholesterol significantly increased (38.17±7.3 % in AD and 28.9±5.8 in normal subjects). The CER/CH ratio was significantly lower (1.53±0.4) in respect to normal skin (2.24±0.61). Our data support the view that impaired biosynthesis of ceramides may be the cause of atopic dry skin and impaired barrier function.

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RESTORATION OF EPIDERMAL LIPID COMPOSITION IN CULTURED SKIN SUBSTITUTES GRAFTED TO ATHYMIC MICE. Jana Vičanová*, Maria Ponec*, M. Dana Harriger#, Steven T. Boyce#, *Department of Dermatology, Leiden University Hospital, Leiden, The Netherlands and #Shrines Burns Institute and #Department of Surgery, University of Cincinnati, Ohio, USA.

Lipids are considered to play an important role in the structure and function of the epidermis, particularly intercellular stratum corneum lipids are necessarily required for optimal stratum corneum barrier function and desquamation. To study restoration of epidermal lipid synthesis, cultured skin substitutes (keratinocytes and fibroblasts attached to a collagen sponge) were incubated in culture, and then grafted onto athymic mice. The analysis of epidermal lipids was performed on samples after 14 and 34 days in vitro, and 3 weeks, 4 and 6 months after grafting. The data show that reconstructed epidermis contains higher levels of triglycerides and lower levels of free fatty acids, cholesterol esters, ceramides and glucosphingolipids compared to values for native human epidermis, with normalization of these lipids by 3 weeks after grafting and complete restoration of the overall lipid profile after 6 months. Fatty acid analysis shows very low levels of linoleic acid (substituted with oleic acid) in vitro that increase after grafting. Similar changes in the overall lipid profile were observed in stratum corneum samples up to 2 years post-grafting. However, ceramide profile in in vitro samples reveals high levels of ceramide 2 and the absence of ceramide 6. At 5 to 11 months post-grafting, the ceramide 6 is present in amounts comparable to native tissue while the level of ceramide 2 still remains high. Complete restoration of ceramide profile was observed at 2 years post-grafting.

These results demonstrate that although cultured keratinocytes do not synthesize all epidermal lipids in vitro, they are capable of restoration of lipogenesis after grafting, suggesting that modulation of culture conditions is expected to improve epidermal lipid profile and barrier function of cultured skin substitutes.

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EXPRESSION OF HUMAN BACTERICIDAL PERMEABILITY INCREASING PROTEIN IN EPIDERMIS.

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We reported that a cysteine proteinase inhibitor, cystatin α, would act as a barrier in the stratum corneum against Staphylococcus aureus V8. Based on this finding, we attempted to characterize other proteins related with epidermal barrier function. In this experiment, the expression of human bactericidal permeability increasing protein (hBPI), which has found in granulocytes and had a high homology with endotoxin binding protein (lipopolysaccharide binding protein), in epidermis is examined. A peptide, whose amino acid sequence was -L-Q-K-E-L-K-R-I-K-I-P-D-Y-S-D-S-F-K-I-K-H-L-G-K-G-C-, was synthesized and conjugated with keyhole limpet hemocyanin (KLH). The hBPI peptide conjugated with KLH (hBPI/KLH) was injected to rabbits to obtain an antibody. In order to investigate its properties, immunoblotting analysis and indirect immunofluorescence technique using anti-hBPI/KLH antibody were performed. A formation of the specific antibody against hBPI was determined by ELISA using the synthesized peptide without KLH as an antigen. Four protein spots in newborn rat epidermal extract of alkaline 10 M urea solution containing 2-mercaptoethanol and NP-40 were reacted with anti-hBPI/KLH antibody, whose isoelectric points were pH 5.3 to 5.5 and molecular weights were about 55 kd. The similar result was observed in human epidermal extract. The immunofluorescent study using newborn rat or human skin revealed that the cell membrane region of the stratum corneum was reacted positively with the antibody. These findings suggest that hBPI finds in both human and newborn rat epidermis and that it locates in the cell membrane region of the stratum corneum.

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DERMAL MICRODIALYSIS ENABLES MEASUREMENT OF PERCUTANEOUS ABSORPTION OF 2-BUTOXYETHANOL IN RAT

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In contrast to inhalative absorption, knowledge about percutaneous absorption of potentially hazardous chemicals is relatively sparse. In this study we used dermal microdialysis techniques to measure kinetics of percutaneous absorption of 2-butoxyethanol (BE), a widely used solvent.

Male Wistar rats were anaesthetized (thiopental) and abdominal hair was clipped. Linear microdialysis membranes (diameter 0.2 mm, cutoff 20 kD) were inserted intracutaneously at a length of 15 mm guided by a canula (25 G) and perfused with isotonic sodium chloride solution at a rate of 15 µl/min. BE (0.3 ml) was applied for one hour in a metal chamber (diameter 1 cm) which was glued on the skin above the membranes. After exposure of one hour the chamber was rinsed with sodium chloride solution three times. Samples of the perfusate were taken before and at the end of exposure and at 1 hour intervals after the exposure for 3 hours.

BE concentration in the perfusate increased during exposure, peaked in the first post-exposure interval (270 µg/ml) and then declined again to reach baseline level about 3 hours after exposure. No increase above the detection limit of 4 µg/ml was found in a non treated control area. From the butoxyacetic acid concentration (main metabolite of BE) measured in urine at the end of the experiment the total amount of BE absorbed during the experiment could be estimated to be about 500 µg.

Dermal microdialysis is an excellent tool for measurement of kinetics of percutaneous absorption. Further studies will be performed on the effect of an impaired barrier function and barrier creams.

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DAYTIME SERUM-LEVELS OF MELATONIN (N-ACETYL-5-METHOXYTRYPTAMINE) AFTER TOPICAL APPLICATION ONTO THE HUMAN SKIN.

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The hormone melatonin is produced in the pineal gland and physiologically underlies a circadian secretion pattern. Due to its antioxidative and immunomodulatory effects it might play a role as a topical drug in future dermatology. However, data on the penetration of topically applied melatonin through the human skin are scarce. In this study we investigated the penetration kinetics of melatonin applied to the scalps of 6 healthy volunteers aged 26-34 years (m/f=2/4). In a first run the individual physiologic daytime melatonin levels were determined with a commercially available radioimmunoassay. In a second run, 3 of the volunteers were treated once with 2 ml of a 1% ethanolic melatonin solution, whereas the other three individuals received 2 ml of 5% melatonin. The topical application was done at 9.00 a.m. and blood samples were collected repeatedly for a total of 8 hours.

Physiologic daytime melatonin levels in the 6 individuals were 16.8 ± 10.0 pg/ml. The three individuals treated with 1% melatonin displayed peak serum levels of 760, 920 and 3400 pg/ml, respectively, whereas in the three volunteers treated with 5% melatonin, peak levels of 1100, 3400 and 4200 pg/ml were measured. The serum levels reached these peak values 1-8 hours after the topical application of melatonin and remained high through the observation period. In contrast to a single oral or intravenous administration where melatonin shows an elimination half-life of less than 1 hour, its topical application was found to result in sustained high serum levels. This effect might be due to accumulation of melatonin in the stratum corneum of the epidermis with continuous liberation from this depot into the blood.

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PROTEASE INHIBITORS PLAY A CRUCIAL ROLE IN HUMAN HORNY LAYER DESQUAMATION. Oliver Wiedow, Claus-W. Franzke, and Enno Christophers, Department of Dermatology, University of Kiel, Germany

Using an in vitro model for plantar callus corneocyte shedding we have detected inhibitory activity for desquamation in extracts of human stratum corneum. Partial purification of these extracts by HPLC revealed two inhibitory activities, which could be identified as antileukoprotease and elafin. Recombinant antileukoprotease was able to almost completely suppress desquamation (96 %) in a concentration dependent manner. Recombinant elafin was shown to exert only weak inhibitory effects on corneocyte shedding. The stratum corneum chymotryptic enzyme (SCCE) represents a serine protease in human stratum corneum which is involved in the process of desquamation. Kinetic analysis of the inhibitory activity of antileukoprotease by kinetic analysis for SCCE revealed a hyperbolic, mixed-type mode of inhibition with an equilibrium dissociation constant of 63.1 nM. This type of inhibition indicates antileukoprotease as potent inhibitor for SCCE with 1:1 stoichiometry at low concentration ranges and a remaining activity (V_∞= 9.8%) of SCCE at indefinitely high concentrations. The inhibitory activity of elafin for SCCE showed a physiological equilibrium dissociation constant of 1.6 µM. In addition the K_i of the two non-toxic inhibitors aprotinin and eglin c was 26.9 and 0.33 nM. Thus, antileukoprotease, which is produced by human keratinocytes, is the most potent human inhibitor of SCCE and desquamation in vitro. It is likely to be involved in the regulation of desquamation under physiological and pathophysiological conditions.

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STRATUM CORNEUM SWELLING

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The aim of this study was to characterize the swelling behaviour of the stratum corneum. Stratum corneum pieces isolated from the breast region from 20 different females were incubated in distilled water, at two different temperatures (20°C and 45°C), for 90 minutes and 24 hours respectively. Half of the stratum corneum pieces had previously been extracted by chloroform:methanol (2:1). The *area*-enlargement was photographically registered. The *thickness*-enlargement was determined using a confocal laser scanning microscope. The average swelling (99% CI) in the area dimension at 20°C was 8.4% ± 1.4% (n=20), which corresponds to an average swelling in the length (lateral) dimension of approximately 4.1%. The swelling in the thickness dimension was 26.3% ± 16.3% (n=8). Our results showed that the swelling is most pronounced in the thickness dimension and that it is completed within 90 minutes of water exposure ($p<0.01$, n=5). In addition, the removal of the intercellular lipids with chloroform/methanol (2:1) induced a decreased swelling in our samples ($p<0.01$, n=20). A raise in temperature of the incubation medium from 20°C to 45°C resulted in an increased swelling ($p<0.01$, n=20). Taken together our results support the idea that the mechanism of stratum corneum swelling is linked to the intercellular lipid structures and hence to skin barrier function.