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001

Frameshift Mutation in the V2 Domain of Keratin 1 Causes the Striate Form of Palmoplantar Keratoderma

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The striate form of palmoplantar keratoderma is a rare autosomal dominant disorder affecting palm and sole skin. Genetic heterogeneity of SPPK has been demonstrated with mutations being characterised in the desmosomal proteins, desmoplakin and desmoglein 1. We have studied a three generation family of British descent with striate PPK in which assessment of affected palmar skin shows a reduced expression of keratins K5/14 in the basal layer and K1/10 and K9 in the spinous layer, as well as up regulation of K6/16/17 across the epidermis. Ultrastructural studies show that desmosome numbers are normal, but their inner plaques and midline structures are attenuated. Microsatellite markers were used to screen candidate loci including the epidermal differentiation complex on 1q, the desmoplakin locus on 6p, the type I and II keratin gene clusters on chromosomes 12q and 17q, and the desmosomal cadherin gene cluster on chromosome 18q. Significant genetic linkage to chromosome 12q was observed using marker D12S368, with a maximum two-point lod score of 3.496 at a recombination fraction of 0. Direct sequencing of the keratin 1 gene revealed a frameshift mutation in exon 9 that leads to the partial loss of the glycine loop motif in the V2 domain and gain of a novel 70 amino acid residue peptide. Using expression studies we show that the V2 domain is essential for the correct insertion of the keratin intermediate filaments into the desmosomal plaque. In conclusion, the ultrastructural findings described here are unique in SPPK, and although the molecular defect is very similar to that recently reported in ichthyosis hystrix the changes in the keratin intermediate filaments are different.

002

E-Selectin Dependent *In Vivo* Migration of Atopic Dermatitis (AD) Derived Th2 Cells Through Chemokine Receptor (CCR) 4 Ligation

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AD is the cutaneous manifestation of atopy and may be induced by allergen specific Th2 cells. Previous data suggest that P- and E-Selectin mediate Th1, but not Th2 cell migration to the skin. In contrast, Th2 cells expressing the E-Selectin ligand cutaneous leukocyte antigen (CLA) dominate early AD lesions and can be isolated from AD skin. CCR3 was initially assigned to Th2 cells, but Th2 cells from AD were not specifically analyzed. In order to understand the multistep process of Th2 cell migration, we investigated CCR expression and E-Selectin binding properties relevant for skin homing of Th2 cells. Highly polarized Th2 clones, grown from AD skin, showed strong CCR4, but no CCR3 or CCR8 expression as determined by real time PCR and FACS analysis. Ca2+ flux assays revealed a selective response to CCR4 ligands macrophage derived chemokine (MDC) and thymus and activation regulated chemokine (TARC) binding, but not to control chemokines such as CCR3 ligand eotaxin or CCR8 ligand I 309. Interestingly, only MDC and not TARC induced complete receptor desensitization indicating also incomplete TARC signaling. Consistently, pronounced expression of MDC was shown in AD skin by *in situ* RT-PCR and superantigen stimulation induced MDC in Th2, but not in Th1 clones. To investigate specific skin homing in a physiologic environment, we developed a SCID-hu mouse model. Human skin was transplanted on SCID mice and AD derived Th2 cells were adoptively transferred (i.p.). Th2 cells migrated exclusively to human skin grafts treated with CCR4 ligands, but interestingly, homing was always more enhanced with MDC than with TARC. This skin homing of human Th2 cells depended on CCR4 ligation, since blocking of G-coupled receptor signaling in Th2 cells by pertussis toxin pretreatment inhibited Th2 cell migration. Also, a blocking E-Selectin mAb strongly reduced Th2 homing, indicating a crucial role for E-Selectin. Our *in vitro* and *in vivo* studies using a SCID-hu Th2 migration model, suggest that rolling on E-Selectin and ligation of CCR4 are functionally relevant steps in Th2 cell skin homing. These data provide insights into the pathogenesis of AD and indicate novel strategies and targets for therapeutic interventions.

003

Platelet Activation in Psoriasis: A Possible Link to Skin Inflammation?

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Circulating lymphocytes gain access to tissues due to their ability to roll along venules. It has been shown that platelet-mediated lymphocyte rolling occurs in lymph nodes: Platelets bind lymphocytes via P-Selectin and lymphocyte P-selectin glycoprotein ligand-1 (PSGL-1). The platelet-lymphocyte-aggregate is then able to roll in a cart wheel-like fashion on the endothelium via platelet P-Selectin and endothelial peripheral node addressin (PNAd). Additionally, in psoriasis increased susceptibility to occlusive vascular disease has been linked to platelet activation. The purpose of this study is to investigate whether platelets mediate lymphocyte homing to skin. If this holds true platelets in psoriatics should express elevated levels of P-selectin and bind lymphocytes. Furthermore, platelets and platelet-lymphocyte aggregates should roll on PNAd-expressing endothelial cells, such as in psoriatic vessels. Platelet P-selectin expression was increased in patients with psoriasis compared to controls (2.1 ± 1.1 vs. $0.7 \pm 0.5\%$; $n = 25$ and 32, respectively) as shown by flow cytometry. In an *in vitro* binding assay, lymphocytes predominantly bound activated platelets (2.4-fold; $n = 6$). Binding of activated platelets is mediated by P-selectin binding to lymphocyte PSGL-1 as it was completely blocked by addition of specific mAbs ($n = 6$). As PNAd is expressed in psoriatic lesions and also constitutively in murine skin ($n = 3$) we observed platelet–endothelial interactions in murine skin vessels using intravital microscopy. For this purpose human platelets were fluorescently labeled, activated, and infused into the carotid artery of anesthetized mice. Fluorescent platelets in downstream microvessels of the ear were visualized by epi-illumination. *In vivo*, rolling of activated platelets along endothelium is significantly increased compared to resting platelets (14.8 ± 11.1 vs. $4.0 \pm 5.3\%$; $p < 0.001$). The increased rolling of activated platelets is abolished by addition of WAPS12.2 (anti human P-selectin mAb) or MECA-79 (anti PNAd mAb) indicating that rolling of activated platelets is mediated via P-selectin–PNAd interaction. We conclude that activated platelets interact with PNAd-expressing skin vessels. This interaction may facilitate rolling of platelet–lymphocyte aggregates as shown by our binding assays. Therefore, platelet activation in psoriasis is hypothesized to aggravate skin inflammatory processes.

004

The G_2/M Regulator 14–3-3 σ Prevents the Progression of Apoptosis by Associating with Bax

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Although the requirement of the p53 tumor suppressor to mediate either cell cycle arrest or apoptosis has been shown, the molecular mechanism by which p53 mediates the balance of either outcome is unclear. 14–3-3 σ (HME1, stratifin) was recently cloned by serial analysis of gene expression as a p53-regulated inhibitor of G_2/M. 14–3-3 σ, which is expressed exclusively in epithelial cells, thereby acts as a central regulator of DNA damage-triggered G_2 arrest. Overexpression of 14–3-3 σ leads to a block of cytokinesis, probably by inhibiting the activation network of the G_2/M cyclin-dependent kinase (CDK) 1, followed by apoptosis. We show that, surprisingly, 14–3-3 σ plays a crucial role in the protection of cells from apoptosis by associating with the pro-apoptotic protein Bax and preventing its translocation from the cytoplasm to membraneous organelles. This quality of 14–3-3 σ is not shared by other 14-3-3 isotypes. Consequently, upon DNA damage parental 14–3-3 $\sigma^{+/+}$ carcinoma cells sustain G_1 and G_2 cell cycle arrest. In contrast, in 14–3-3 $\sigma^{-/-}$ cells DNA damage leads to a dramatic translocation of Bax and distinct concentration of mitochondria and Bax around the centrosome, preceeding activation of caspase-3, and ultimately apoptosis, as judged by sub-G_1 peak, DNA laddering, and annexin V stain. This scenario is further accompanied by caspase-dependent cleavage of the CDK inhibitor $p21^{CIP1}$, resulting in its functional inactivation through cytoplasmic translocation, unavailability for G_1 arrest, and lack of CDK2 inactivation. In conclusion, 14–3-3 σ not only enforces the p53-dependent G_2 arrest but also delays the apoptotic signal transduction by associating with Bax. Therefore, strategies to target 14–3-3 σ during chemotherapy might contribute to the therapeutic efficiency.

005

A Novel Approach to Characterise the BP180 Epitope Pattern Recognised by IgG Autoantibodies of Bullous Pemphigoid (BP) Patient Sera

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The BP180 antigen, a glycoprotein transmembrane component of the epidermal hemidesmosome, is recognised by autoantibodies associated with three autoimmune bullous skin disorders: BP, herpes gestationis and cicatricial pemphigoid. An immunodominant region of BP180 recognised by sera of BP patients has been mapped to the membrane-proximal noncollagenous stretch (NC16A) of the BP180 ectodomain. However, BP autoantibodies reactivity to BP180 is not restricted to NC16A region but also comprises epitopes located in the intracellular and others extracellular domains. In order to characterise the targeted antigenic sites on the whole BP180 antigen in BP, we have prepared a random BP180 epitope library on bacteriophage λ and selected it with sera from BP patients. BP180 cDNA has been randomly digested and cloned in λ as a D protein fusion on the bacteriophage capsid. At first, the library has been screened by affinity selection using three anti-BP180 monoclonal antibodies (mAb). The genome of the selected recombinant phages bearing BP180 fragments has been sequenced and the minimal recognised regions mapped. The known BP180 antigenic segments for 1A8c, 233 and 1D1 mAb were reduced, respectively, to regions of 6 (residues 155–160), 26 (residues 1118–1143) and 31 (residues 1357–1387) aminoacids indicating the potential of this λ library to map epitope pattern of BP patients sera. The λ library has then been selected with sera from 10 BP patients and several epitopes spread over the entire BP180 molecule have been identified. The reactivity of 35 BP patient sera has been assessed against 10 selected epitopes of BP180 intracellular and extracellular regions. Our results show that phage display libraries represent an alternative approach to epitope mapping of humoral response in autoimmune blistering skin diseases. This technique might also be used to characterise epitope spreading in disease course, to correlate the clinical features to epitope pattern and to set up a diagnostic ELISA.

006

The Laminin γ2 Chain Short Arm Drives Incorporation of Laminin-5 into the Extracellular Matrix, Sustains Cell Adhesion and Inhibits Cell Migration

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Laminin-5 is a basement membrane component that actively promotes adhesion and migration of epithelial cells. Laminin-5 undergoes extracellular proteolysis of the γ2 chain that removes the NH_2-terminal short arm of the polypeptide and reduces the size of laminin-5 from 440 kDa to 400 kDa. The functional consequence of this event remains obscure although lines of evidence indicate that cleavage of the γ2 chain potently stimulated scattering and migration of keratinocytes and cancer cells. To define the biological role of the γ2 chain short-arm, we generated mutant γ2 cDNAs that were transfected into the immortalized γ2-null cell line LSV5. Mutant pγNC expresses a recombinant γ2 polypeptide with the internal deletion of amino acid sequence (YSGD) that constitutes the proteolytic cleavage site of the γ2 chain. Mutant pγGP modifies the configuration of the γ2 chain domain III. Mutants pγIII and pγV carry a deletion affecting the EGF-like repeat 1 of domain III and repeats 2 and 3 of domain V, respectively. Mutant pγC encodes a polypeptide lacking the N-terminal domains IV and V that are excised in the extracellular processing of laminin-5. Mutant pγM corresponds to a mutated γ2 chain, missing 73 amino acids of the short arm, that causes a mild form of junctional epidermolysis bullosa, a recessive inherited skin blistering disease. By immunofluorescence and immunohistochemical studies, cell detachment and adhesion assays, we found that the γ2 short arm drives deposition of laminin-5 into the extracellular matrix ECM and sustains cell adhesion. Our results demonstrate that the unprocessed 440-kDa form of laminin-5 is a biologically active adhesion ligand, and that the γ2 globular domain IV is involved in intermolecular interactions that mediate integration of laminin-5 in the ECM and cell attachment.

007

Leptin Modulates the Effects of DGAT Deficiency on Fur and Sebaceous Glands in Mice

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Acyl:CoA diacylglycerol acyltransferase (DGAT), the enzyme that catalyzes the final step in triglyceride synthesis, is highly expressed in sebaceous glands of mice. DGAT-deficient ($Dgat^{-/-}$) mice developed sebaceous gland atrophy after puberty, resulting in the production of sebum that lacked several normal components (including a putative type II wax diester, a major constituent of mouse sebum). These abnormalities were associated with defective water repulsion and prolonged hypothermia in $Dgat^{-/-}$ mice after water immersion. Unexpectedly, the histological and functional abnormalities observed in $Dgat^{-/-}$ mice required the presence of leptin, because leptin-deficient (*ob/ob*) mice with DGAT deficiency had detectable sebaceous glands and produced normal sebum. However, 2 weeks of subcutaneous leptin infusion (250 ng per h, a dose that restores normal plasma leptin levels) led to sebaceous gland atrophy and abnormal sebum composition in $Dgat^{-/-}$ *ob/ob* mice, and these findings reverted 2 weeks after the completion of leptin infusion. Intracerebroventricular leptin infusion (10 ng per h, a dose that does not increase circulating leptin levels in the periphery) produced similar results, indicating that the effects of leptin on sebaceous glands in $Dgat^{-/-}$ mice could be mediated entirely through the central nervous system. Our findings identify DGAT as a key enzyme in the development and functioning of sebaceous glands and reveal a newly identified relationship between sebaceous glands and leptin that is mediated through the central nervous system.

008

Comparison of Genetic with Conventional Protein Vaccines: Induction of Protective Antitumor T Cell Responses Against Tumor Antigen Expressed by pDNA-Based Vaccine but not Against Recombinant Protein

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The recent development of genetic vaccines raises the question whether these genetic vaccines are superior to conventional immunization strategies employing the respective protein for the induction of protective T cell responses. We therefore cloned the 1.9 kb full length cDNA murine homologue of human MAA Pmel17/gp100, generated a recombinant baculovirus containing the respective cDNA sequence by ligation independent cloning, expressed the recombinant protein in Sf9 insect cells, and confirmed its identity by SDS-PAGE, Western blot analysis and ELISA with Pmel17 reactive Ab HMB45. After large scale purification by preparative SDS-PAGE, electroelution, dialysis and steril filtration this recombinant full-length protein was compared with a pDNA vaccine for the induction of protective antigen-specific T cell responses in the experimental Cloudman M3/DBA/2 mouse melanoma model. Mice genetically immunized with mPmel17-encoding pDNA showed significant protection against subsequent tumor challenge with $Pmel17^{+}$-M3 melanoma cells in contrast to mice immunized with increasing doses of recombinant protein ($p < 0.001$ at day 15). Whereas induction of an antigen-specific T cell response by mPmel17-encoding pDNA could be demonstrated by (i) induction of protection against $Pmel17^{+}$- but not $Pmel17^{-}$ M3 melanoma sublines, (ii) complete loss of protection by *in vivo* $CD4^{+}/CD8^{+}$ T cell depletion and (iii) the detection of a Pmel17/gp100-specific T cell response in CTL assays antigen-specific T cell responses could not be detected in mice immunized with the respective recombinant protein. These results demonstrate for the first time that i.c. genetic immunization with antigen-encoding pDNA is superior to immunization with the respective recombinant protein for the induction of antigen-specific, protective antitumor T cell responses *in vivo*.

009

Human Cathelicidin Antimicrobial Peptide is Induced in Skin Wounding – Active Peptide is Detected in Physiologic Healing But Not in Chronic Nonhealing Wounds

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The antimicrobial peptide hCAP18 is a key effector molecule in innate immunity. hCAP18 consists of a conserved propart, cathelin, and a variable C-terminus called LL-37 in humans. The peptide was originally isolated from neutrophils and is up-regulated in skin in response to inflammation. The holoprotein is activated by enzymatic processing, releasing the LL-37 peptide that confers the antimicrobial effects. To further explore its role in barrier protection, we have studied hCAP18 in human wound healing. In both chronic nonhealing wounds and in physiological wound healing, there was intense immunoreactvity for hCAP18 in the inflammatory wound bed infiltrate. Low levels of myeloperoxidase protein, measured with ELISA, were found in protein extracts from all wounds, indicating only a minimal neutrophil contribution to hCAP18 in these tissues. By *in situ* hybridization the main signal for hCAP18 was detected in the basal layer of the epidermal tounge near the wound edge. In physiologic healing, hCAP18 was rapidly up-regulated attaining maximum levels at 12 h postwounding and declining to basal prewounding levels at 14 days. hCAP18 protein levels in chronic wounds were only 20% of maximum levels detected in the physiologically healing wounds. Furthermore, with Western blot, processed active peptide was only seen in these latter wounds and not in the chronic wounds. In our noninflammatory ex-vivo wound healing model, hCAP18 was up-regulated in the migrating keratinocytes persisting at re-epithelialization by days 5–7, indicating that inflammation is not required for induction of hCAP18 gene expression. hCAP18 is the only cathelin protein in human, but the porcine protein, PR-39, a member of the same gene family, is known to induce syndecans mediating growth-factor responses in pig wounds. The precise role for hCAP18 in wound healing remains to be fully established, however, the lack of active LL-37 peptide in chronic nonhealing wounds may contribute to the impaired healing process.

010

Adoptive Immunity Does Not Modulate Clonal Expansion of UV-Induced p53-Mutant Clones or Their Early Phase Regression

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The P53 tumor suppressor protein has a central role in the development of UV induced sqamous cell carcinomas in human and mouse. P53 protein is not expressed in immunohistochemically detectable amounts in the epidermis in normal conditions. If the half-life of the protein is increased due to the regulation or mutation it can be detected by immunoperoxidase staining. According to previous studies clusters of keratinocytes staining positive for p53 can be found after chronic UVB irradiation. Most of these p53 positive clones harbor p53 mutations and might represent precancerous lesions. The kinetics of clone development and regression are not well understood. To determine whether the immune system has an effect on the formation or elimination of p53 immunopositive clones, immunodeficient mice were used. Mice in which the RAG-1 gene has been knocked out are deficient in adoptive immune response due to loss of the recombinase required for B and T cell development. Shaved RAG-1 KO and corresponding C57BL/6 WT mice were irradiated with 750 J per m^2 UVB daily 5 times per week for 7–11 weeks. Groups of mice were euthanized at 7, 9, 11 weeks. UVB treatment was stopped at 7 and 9 weeks for another two groups of mice which were kept alive without UVB for 2 more weeks. Whole mounts of epidermis were prepared and immunoperoxidase staining was performed using CM5 polyclonal antibody. The number of p53 positive clones per area and the number of cells/clone were counted under the microscope and statistically analyzed. The number of clones per area increased with irradiation time. After longer irradiation there were more large clones. Statistical analysis revealed no significant difference in clones per area between WT and RAG-1 KO mice at 7, 9 or 11 weeks. 2 weeks after the UV irradiation was stopped on the 7th or 9th week, the number of clones had decreased in both WT and KO mice without significant difference between the two groups. Unirradiated WT and KO mice had no p53 positive clones. Our results indicate that (a) UV light is necessary to produce clones, (b) longer irradiation time increases the number and size of clones, (c) without UV light the clones showed regression, (d) the adoptive, antigen specific, immunity is not involved in preventing the development of p53 clones, and (e) adoptive immunity is not involved in the early regression of p53 positive clones. The innate immunity, keratinocyte apoptosis, and the epidermal turnover may contribute to the regression of the clones.

011

The Signaling Function of Integrin β4 Requires Palmitoylation of the Protein

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Integrin α6β4 is a major receptor for the adhesion ligand laminin-5. Its interaction with laminin-5 leads to the formation of hemidesmosome in stratified epithelia. We have previously shown that α6β4 can activate the Ras-Erk signalling pathway by direct recruitment of the signaling protein Shc to the tyrosine phosphorylated cytoplasmic domain of β4. Shc is an SH2-PTB domain adaptor which links various tyrosine kinases to Ras by recruiting the Grb2/mSOS complex to the plasma membrane. Shc activation is essential for a normal growth of epithelial cells such as keratinocytes. Here we report that the β4 cytoplasmic domain is covalently modified by the post-translational addition of a palmitic acid on a cysteine residue, and that palmitoylation is differentially modulated in a variety of cell lines. By directed mutagenesis of the β4 cytoplasmic domain we show that palmitoylation occurs close to the transmembrane domain of the polypeptide. By sucrose gradient floatation assay we also show that β4 partially localizes into rafts, specific microdomains of the plasma membrane enriched in cholesterol and glycosphingolipids, and that such a localization is dependent on palmitoylation. We also demonstrated that palmitoylation-deficient β4 mutant preserves the adhesion capacity and ability to assemble hemidesmosomes. However, by transient tranfection assays in HUVECs cells, we show that this mutant does not signal to ERK and that the Src familly kinase are essential for ERK phosphorylation in response to β4 ligation. In addition, we assess that the fraction of β4 found in the rafts associates with a Src family kinase which is enriched in the rafts, possibly Yes or Fyn. Our data suggest that the absence of ERK phosphorylation correlates with the inability of the palmitoylation-deficient β4 to localize into rafts and to interact with a Src kinase, which indicates that rafts play a crucial role in the signalling pathways of the integrin α6β4.

012

Development of an Active Mouse Model of Pemphigus Vulgaris, a Life Threatening Skin Disease, by DNA Immunization

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Pemphigus Vulagaris (PV) is a life threatening skin disease mediated by IgG antibodies to desmoglein-3 (Dsg3), a member of cadherin supergene family of cell-cell adhesion molecules. Development of an animal model of PV is crucial for development of novel approaches in antigen-specific immunotherapy of the disease. We here present our data on successful development of PV-like lesions in a specific strain of mouse by DNA immunization. cDNA coding for the full length mouse Dsg3 was conjugated with Flag sequence and subcloned into a mammalian expression vector under a CMV promoter. The resulting construct, using *in vitro* transfection studies, was shown to express the full length Dsg3 as confirmed by Western blot using anti-Flag and anti-Dsg3 antibodies. The endotoxin free cDNA was then delivered by particle bomardement. The mice developed anti-Dsg3 autoantibodies as detected by immunofluorescence microscopy, Elisa and Western blot. Clinically, the mice developed hair loss, and mucosal lesions. Adoptive transfer of splenocytes from affected mice into SCID mice further demonstrated the ability of autoreactive lymphocytes in development of PV-like lesions. This is the first demonstration of an active mouse model of PV by DNA immunization. This model would provide an invaluable tool for exploring the immunopathology of PV and development of novel immunotherapeutical approaches.

013

Ribozyme Gene Therapy for Keratin Disorders

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There are now 17 keratin genes that are known to be involved in autosomal dominant genodermatoses and other inherited epithelial disorders. Due to the dominant-negative effect of mutant keratins, conventional gene replacement therapy is not appropriate for these diseases. Ribozymes are small catalytic RNA molecules which can be designed to cleave mRNA molecules at specific sites. A total of 19 potential ribozyme sites in the K14 mRNA were evaluated and constructs were made for the five sites showing best predicted RNA folding characteristics. Ribozymes were protected from RNAse degradation *in vivo* by addition of hairpin sequences derived from the U1 snRNA, a naturally occurring small RNA molecule that escapes nuclease attack. Ribozymes directed against the five chosen sites were constructed as mini-genes driven by the U1 snRNA promoter in a modified plasmid vector. The ability of these ribozymes to cleave K14 RNA was investigated under a wide range of conditions both *in vitro* and in cultured cells. Three ribozymes were capable of cleaving K14 mRNA *in vitro*. Furthermore, two were capable of rapidly cleaving more than 90% of K14 mRNA following transfection of the ribozyme plasmids into cultured keratinocytes. These experiments show (a) that ribozymes are capable of specifically degrading even high-abundance mRNA species such as keratins; and (b) pave the way for gene therapy where all endogenous keratin mRNA might be ablated and replaced by a modified ''ribozyme-immune'' keratin gene. In addition, it should be possible to develop ribozymes that target specific keratin mutations.

014

Proteasome Inhibitor PS519 Reduces Superantigen-Mediated T-Cell Activation and Severity of Psoriasis in a SCID-Hu Model

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Psoriasis is a T-cell mediated skin disease characterized by the infiltration of activated leukocytes and the presence of a distinct inflammatory pattern. There is increasing evidence that bacterial superantigen-mediated activation of T-cells plays a pivotal role in the induction of psoriasis. NF-κB is a transcription factor which is involved in the induction of many pro-inflammatory molecules known to be up-regulated in psoriasis. We therefore addressed the question whether attenuation of NF-κB activation by the proteasome inhibitor PS-519 may suppress T-cell mediated responses and may be effective in the treatment of psoriasis. PBMCs of four healthy volunteers were stimulated with the superantigen TSST-1 (100 ng per mL) in the absence or presence of nontoxic concentrations of PS-519 (1–10 μg per mL). We found a dose-dependent inhibition of lymphocyte proliferation of up to 95% compared to TSST-1 treated cells. Additionally, the expression of T-cell activation markers such as CD69, CD25 and HLA-DR was markedly reduced in PS-519 treated, TSST-1 stimulated cultures. Cytokine production by superantigen-stimulated PBMCs was dramatically and significantly reduced in PS519 treated cultures (IL-1β, TNF-α, IFNγ). The effects of PS-519 on the severity of psoriasis was assessed in a xenogenic SCID-hu transplantation model. Lesional psoriatic skin obtained from different donors was transplanted onto SCID mice. After 28 days, SCID mice were treated once daily either with PS-519 intraperitoneally (20 μg per mouse; $n = 8$) or with vehicle for 28 days ($n = 4$). Thereafter, 20S proteasome activity was determined in the blood and the human transplanted skin examined by immunohistochemistry. As expected, 20S proteasome activity was markedly reduced in PS-519 treated mice (0.22 ± 0.01 [pmol AMC per s per mg protein]) compared to untreated mice (1.53 ± 0.05; mean ± SEM). In PS-519 treated grafts, normalization of epidermal architecture including loss of papillomatosis and marked reduction of akanthosis was observed. We conclude that the proteasome inhibitor PS-519 reduces superantigen mediated T-cell activation *in vitro* and shows antipsoriatic efficacy in the SCID-hu model.

015

Psoriasin (S100a7) is a Major and Potent *E. coli*-Selective Antimicrobial Protein of Healthy Human Skin

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Human skin is markedly resistant towards infection by Gram-negative bacteria, in particular *E. coli*. We therefore hypothesised that human skin might contain factors that control *E. coli* growth. In order to prove this hypothesis we analysed protein extracts obtained from healthy donors heel stratum corneum and psoriatic scales for the presence of *E. coli* killing factors using the radial plate diffusion antimicrobial assay system with *E. coli* as read-out-system. Heparin-sepharose-HPLC followed by preparative reversed-phase- and cation-exchange-HPLC revealed microheterogeneous microbicidal 10 kDa proteins by SDS-PAGE that could be purified from both, heel callus and psoriatic scales, in hundreds of microgram amounts. All isolated 10 kDa proteins showed a blocked NH_2-terminus. Peptide mapping with subsequent protein sequencing as well as electrospray-ionisation-mass-spectrometry revealed all proteins to be identical with post-translationally modified forms of the S100-protein psoriasin. The predominant psoriasin variants showed at physiologic concentrations in the microdilution assay system bactericidal activity for *E. coli* ($LD_{90} < 10$ μg per mL), not however, for *P. aeruginosa*, *S. aureus* and *C. albicans* at concentrations below 200 μg per mL, indicating a specificity for *E. coli*. Using realtime-RT-PCR with intron-spanning primer pairs we found psoriasin mRNA unexpectedly to be expressed at a high level in healthy skin, respiratory epithelia and normal primary keratinocyte cultures. Because psoriasin protein is nearly 10-fold more abundant in psoriatic scales vs. healthy donors callus, we speculated that psoriasin expression – in contrast to previous suggestions – is inducible. To prove this hypothesis we investigated mRNA-levels in primary keratinocytes after treatment with various inflammatory cytokines. Psoriasin-mRNA was induced 16 h after stimulation with either TNF-α, both IL-1-forms and IL-6. Furthermore, the contact with various microorganisms induced psoriasin-mRNA with the mucoid form of *P. aeruginosa* being the most potent one. In order to elucidate the mechanism how *E. coli* is killed by psoriasin, we also analysed psoriasin-treated *E. coli* ultrastructurally. Unlike the treatment with defensins no signs of membrane alteration were seen, which indicates that psoriasin kills *E. coli* by a unique mechanism. In conclusion, our findings demonstrate that a major biological function of psoriasin might be to prevent infections of the skin with *E. coli*.

016

Interleukin-12 Suppresses Ultraviolet Radiation-Induced Apoptosis by Inducing DNA Repair

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We recently observed that the immunomodulatory cytokine interleukin 12 (IL-12) protects keratinocytes from ultraviolet radiation (UV)-induced apoptosis *in vitro*. Accordingly, i.c. injection of IL-12 into mice before UV exposure suppresses the formation of sunburn cells. Interestingly, UV-induced DNA damage was found significantly reduced both *in vitro* and vivo when DNA was extracted several hours after UV exposure. In contrast when DNA was extracted immediately after UV exposure, the amounts of UV-induced DNA lesions were the same irrespective whether IL-12 was applied or not. This implies that IL-12 removes UV-induced DNA lesions by inducing DNA repair. Accordingly, expression of a UV-damaged chloramophenicol acetyltransferase expression vector was significantly induced when transfected into cells pretreated with IL-12, compared with diluent treated cells. These findings were confirmed by the comet assay. Comet formation which is not only an indicator for the severity of DNA damage but also for the efficacy of incision during nucleotide excision repair was induced by IL-12, indicating that IL-12 induces DNA repair. Accordingly, RNase protection assay revealed that IL-12 induces the expression of particular components of the nucleotide excision repair complex. The enhanced repair capacity induced by IL-12 improved cell survival as demonstrated by enhanced colony-forming ability of both transformed and normal human kerationcytes after UV irradiation. Taken together, this study for the first time demonstrates (i) that cytokines can protect cells from apoptosis induced by DNA damaging UV radiation via inducing DNA repair and (ii) that nucleotide excision repair can be manipulated by cytokines.

017

Collagen XVII is Processed by TACE and Other Sheddases

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Collagen XVII is a structural component of the hemidesmosomes and exists in two forms, as 180 kDa type II transmembrane protein and as soluble 120 kDa ectodomain, which corresponds to the extracellular domain of collagen XVII. We have previously shown that the ectodomain was generated by proteases, which were subjected to a furin-mediated process. In this work we identified the enzymes, which release the ectodomain from the cell surface. Time chase experiments with biotinylated cultured keratinocytes showed that the soluble ectodomain was stable in the medium for more than 48 h. The use of domain specific antibodies demonstrates that the authentic shedding product contains at least a part of the NC16A domain and the C-terminus of the collagen XVII molecule. Collagen XVII-shedding of cultured keratinocytes was enhanced by phorbol esters and inhibited by phenanthroline, MMP- and sheddase-targeting hydroxamates and TIMP-3, but not by TIMP-1, TIMP-2, a selective gelatinase inhibitor and serine protease inhibitors. The candidate enzymes MMP-2, MMP-9 and MT1-MMP, which are involved in proteolytic cascades on keratinocyte surfaces, were excluded, since they cleaved purified collagen XVII to a nonphysiological fragment and MMP-2 and MT1-MMP-deficient cells showed normal collagen XVII-shedding. Promising candidate enzymes are members of the ADAMs, like the prototype sheddase TACE, ADAM-10 and ADAM-9. All these sheddases contain a putative furin-activation site in their molecule. RT-PCR analysis and immunoblotting demonstrate that TACE, ADAM-10 and ADAM-9 were expressed and activated in human keratinocytes. Immunohistological analysis of human skin revealed that especially TACE- and ADAM-9 were mainly restricted to basal keratinocytes and therefore showed a localization comparable with collagen XVII. To verify the role of TACE in collagen XVII-shedding, HaCaT cells were transfected with full-length murine TACE-cDNA. A dose dependent increase of ectodomain-shedding, with a concomitant decrease of full-length collagen XVII were shown. In addition, TACE-deficient murine keratinocytes showed significantly reduced collagen XVII-shedding. The results support the conclusion that TACE contributes to collagen XVII-shedding from the keratinocyte surface. Further studies will determine the identity of the other sheddases, which are also involved in this process.

018

Identification of ERBIN, a Novel LAP/LERP Protein, as Interaction Partner of the Hemidesmosomal Proteins Bullous Pemphigoid Antigen 1 and the Integrin β4 Subunit

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The bullous pemphigoid antigen 1 (eBPAG1) is a component of hemidesmosomes (HD), junctional adhesion complexes in stratified epithelia. While its COOH-terminus interacts with intermediate filaments, its NH_2-terminus is important for its localization into HD. To identify proteins which interact with the NH_2-terminus of human eBPAG1, we performed a yeast two-hybrid screen, which uncovered a novel protein belonging to the LAP/LERP (for *LRR and PDZ* domain) protein family with 16 NH_2-terminal leucine rich repeats (LRR) and a COOH-terminal PDZ domain. The gene for this LAP/LERP protein comprises at least 26 exons located on the long arm of chromosome 5. In most human tissues, several transcripts were detected differing in the coding region situated upstream of or within the PDZ domain. One of the encoded variants was found to correspond to the recently described protein ERBIN. In yeast and in *in vitro* binding experiments ERBIN was shown to interact not only with eBPAG1, but also with the COOH-terminal region of the cytoplasmic domain of the integrin β4 subunit, another component of HD. Antibodies raised against the COOH-terminus showed that ERBIN is expressed in keratinocytes. In transfected epithelial cells the protein, however, was not localized in HD but either diffusely distributed over the cytoplasm or concentrated at the basolateral plasma membrane. Since ERBIN has previously been shown to interact with the transmembrane tyrosine kinase receptor Erb-B2, which in turn associates with the integrin β4 subunit, we suggest that ERBIN provides a link between HD assembly and Erb-B2 receptor signaling.

019

Increased Expression of Human Toll-Receptor Homologues by Human Keratinocytes may Contribute to the Activation of Innate Immunity in Infected Skin

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The ability to sense the presence of pathogenic organisms is part of the active role of human keratinocytes in mounting an innate immune response resulting in the release of antimicrobial peptides which kill invading microorganisms. To explain our findings of epithelial peptide antibiotic β-defensins 2 and 3 (hBD-2, hBD-3) induction after contact with microorganisms, we speculated about a role of keratinocyte-derived pathogen pattern recognition receptors. Using semiquantitative and real-time RT-PCR with gene specific primers we detected mRNA expression of human Toll-like receptors (hTLRs) in cultured primary human keratinocytes (NHK) and the keratinocyte cell line HaCaT. While hTLR 1 and 7 mRNA species seem to be expressed in HaCaT keratinocytes at a low level (after stimulation with heat inactivated *Pseudomonas aeruginosa*), stronger RT-PCR signals were found for hTLR 2–6. Interestingly, upon incubation with heat inactivated *Pseudomonas aeruginosa* or *Staphylococcus aureus*, we found up-regulated expression of mRNA encoding Toll-like receptors hTLR2 and hTLR4 in NHK and HaCaT cells. Relative induction of hTLR4 was found most prominent in NHK and after keratinocyte contact with heat killed Gram-negative *Pseudomonas* microorganisms. Increased expression of and signalling by hTLR2 (with lipoteichoic acids derived from Gram-positive bacteria as ligands) and/or hTLR4 (with lipopolysaccharides from Gram-negative bacteria as ligands), and perhaps other Toll-receptor homologues, may contribute to the activation and maintenance of innate immunity in infected skin.

020

Interleukin-18 is a Key Proximal Mediator in the Regulation of Langerhans Cell Migration and Contact Hypersensitivity in Mice

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Langerhans cell (LC) migration from the epidermis to regional lymph nodes is a tightly regulated process, and current data suggest that interleukin (IL)-1β and tumour necrosis factor-α (TNF-α) are involved. In the present investigations we have assessed the role of IL-18, a cytokine structurally similar to IL-1β, in the regulation of LC migration and contact hypersensitivity (CHS) utilising IL-18 knock-out (KO) mice. To determine whether IL-18 is required for optimal contact sensitisation, IL-18 KO and wild type (WT) mice were sensitised on abdominal skin with 1% oxazolone (OX) and challenged on the dorsum of one ear 5 days later with 0.5% OX. Vigorous ear swelling responses were observed in WT mice, but this response was inhibited by 36% ($n = 3$; $p < 0.05$) in IL-18 KO mice. This attenuated response was completely restored in IL-18 KO mice by local intradermal injection of IL-18 (50 ng) immediately prior to sensitisation with OX ($n = 3$) suggesting that the defect in IL-18 KO mice was in the afferent phase of the CHS response. Identical administration of IL-18 to WT mice did not affect the CHS response. To examine the effect of IL-18 on allergen-induced LC migration, epidermal LC density was determined following topical application of 1% OX. OX treatment caused a significant decline in MHC class II$^+$ epidermal LC density 4 h after application in WT mice (26%, $n = 3$; $p < 0.05$) but this was absent in IL-18 KO mice. Intradermal injection of exogenous IL-1β, TNF-α or IL-18 (50 ng each, $n = 3$) lead to equivalent LC migration in both IL-18 KO and WT mice, indicating that, given an appropriate signal, IL-18 KO LC are able to migrate normally. Finally, intradermal injection of IL-18 prior to topical sensitisation with 1% OX in caspase-1 deficient mice (which have impaired release of both IL-1β and IL-18) failed to restore the attenuated CHS response, whereas IL-1β pretreatment resulted in complete restoration ($n = 3$). These results indicate that IL-18 is a key proximal mediator of LC migration and CHS, acting upstream of IL-1β, and that this cytokine may play a central role in the regulation of cutaneous immune responses.

021

APC-Independent T Cell Activation of Hapten-Specific CD4$^+$ T Cells

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Dendritic cells (DCs) are crucial for the initiation of allergic contact dermatitis (ACD) to haptens. However, the expression of ACD is largely DC-independent, and the mechanisms responsible for T lymphocyte activation at the site of hapten challenge have not been fully elucidated. We have investigated the APC requirement of nickel (Ni)-specific CD4$^+$ lymphocytes isolated from the blood of 6 allergic individuals. 42 out of 121 (35%) T cell clones (Tccs) proliferated *in vitro* to Ni in the absence of professional APCs, suggesting a direct T-T Ni-presentation. Ni recognition by APC-independent clones was MHC class II restricted, was not influenced by CD28 triggering, and occurred independently from the activation state of the Tccs. Additionally, the epitope recognized by these Tccs did not require processing, as indicated by experiments performed with fixed APCs. APC-independent and -dependent Tccs expressed similar levels of MHC class II and B7 molecules, and did not differ in their antigen presenting capacity, since they both induced comparable activation of APC-independent, but not -dependent Tccs. T-T presentation induced prominent CD3/TCR down-regulation, CD25 up-regulation and IFN-γ release, although to a lesser extent compared to those induced by DCs. Finally, T-T presentation was not followed by T cell anergy, as indicated by the capacity of T cells to respond to the hapten after multiple cycles of activation with Ni in the absence of professional APCs. Our data suggest that APC-independent T cell activation could represent an important mechanism for the initiation and amplification of ACD.

022

The Role of T-T–Cell Interactions for Differentiation of Antigen-Specific Helper T Cells *In Vivo*

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During stimulation with antigen naive CD4$^+$ T helper (Th) cells acquire the capacity to produce large amounts of cytokines. During this priming Th cells tend to differentiate into polarized interferon γ (IFNγ) producing Th1 or IL-4 producing Th2 cells. Even though mechanisms regulating Th1 and Th2 differentiation *in vitro* are well described, the initial event responsible for the Th1 or Th2 differentiation *in vitro* remains open. Importantly, the primary source providing the IL-4 responsible for T cell differentiation toward a Th2 phenotype is unknown. Here we asked whether differentiated memory cells could provide this IL-4 during T-T–cell interactions *in vivo*. To address this question we established an *in vivo* system, using Th cells from transgenic mice with TCR specific for either the OVA or the HA peptide. Following the adoptive transfer of naive HA-Th cells or fully differentiated OVA-Th2 cells, we determined whether OVA-Th2 cells could influence the differentiation of the naive HA-Th cells, when stimulated with specific peptide and Complete Freund Adjuvans (CFA), which strongly promotes Th1 development. Both, naive and Th2 memory T cells migrated to draining lymph nodes and spleen, even in absence of a specific stimulus. Following injection of both, antigen and peptide, the numbers of specific Th cells increased again, in nodes and the spleen. Importantly, the presence of Th2 memory T cells alone did not impact the *in vivo* differentiation of naive HA-Th cells, stimulated with peptide and CFA. However, when both Th cell populations were activated simultaneously, OVA-Th2 deviated the phenotype of the freshly activated HA-Th cells toward a Th2 phenotype, directly showing that activated Th2 populations can instruct the differentiation of concomitantly activated naive Th cells. This may contribute to the spreading of Th2 immunity, frequently observed during the development of atopic diseases.

023

Central Role of CD4$^+$ T Cells in the Psoriasiform Skin Disease of CD18 Hypomorphic Mice

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Recently we reported a polygenic mouse model in the PL/J strain resembling human psoriasis which – due to a hypomorphic mutation in the CD18 gene – reveals a severe reduction of CD18 (β2-integrin) expression to 10% of PL/J wild type mice. Immunostainings revealed a significant increase ($p < 0.0001$) in CD4$^+$ T cells, CD8$^+$ T cells and macrophages in CD18 hypomorphic compared to wild type mice ($n = 3$). Since CD4$^+$ T cells have been suggested to play a key role in a variety of inflammatory processes either via initiation and amplification of CD8$^+$ cytotoxic effector functions or recruitment and stimulation of macrophages with keratinocyte proliferation and CD4$^+$ T cell stimulating properties, we have used CD4$^+$ T cell depleting antibodies *in vivo* to study the pathogenic role of CD18 hypomorphic mice. I.p. administration of the affinity purified monoclonal rat anti mouse antibody (GK1.5) at a concentration of 150 μg 3 times a week resulted in an almost complete reduction (> 95%) of circulating and skin localized CD4$^+$ cells as judged by FACS analysis and immunohistochemistry. Using an adapted PASI score, we found a complete resolution in two (10–1) and an almost complete resolution in 1 animal (9–2). Similarly affected CD18 hypomorphic mice treated with CD8$^+$ depleting or isotype matched control antibodies did not show any improvement of the PASI score, suggesting a central CD8 ± independent role of CD4$^+$ T cells in the pathogenesis of the psoriasiform skin disease. As CD18 expression has been reported to be significantly reduced in psoriasis patients and in order to study whether the level of CD18 expression has an impact on the development and severity of the disease, we have generated a PL/J mouse line which – in contrast to the herein used PL/J hypomorphic CD18 mice – reveal a complete deficiency in CD18 (CD18 null mutation). Interestingly, the PL/J CD18 null mice did neither develop any psoriasiform skin disease nor any CD4$^+$ skin infiltration during an observation period of > 2 years supporting the view that the pathogenic involvement of CD4$^+$ T cells in the skin disorder of the PL/J CD18 hypomorphic mice depends on a gene dose effect with reduced expression of the CD18 protein.

024

Critical Role of CD18 in Lymphoid Tissue Organization and T-Dependent B Cell Response

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β_2 integrins play a central role in host defence and if specifically mutated lead to a disorder known as leukocyte adhesion deficiency 1 (LAD1) in humans. Mice with a CD18 null mutation suffer from recurrent bacterial infections, impaired wound healing and skin ulcers closely resembling human LAD1. We here report on the impact of CD18 deficiency on the organization of lymphoid tissue, B cell differentiation, germinal center formation and T-dependent B cell response. CD18 null mice show a complex B cell phenotype including a severe increase in plasma cells leading to a 10- to 20-fold elvated serum IgG and light chain amyloid deposits. Furthermore we detected a significant increase in serum interleukin-6 levels. Since IL-6 is a potent enhancer of plasma cell formation and immunoglobulin secretion, we have established a CD18xIL-6 double-null mouse model in which the bias of IL-6 on overall antibody production was destinctly abolished, as we could show by IgG serum levels that were reduced to those of wild type (wt). Subsequently, CD18 single deficient, CD18xIL-6 double-null and wt control mice were immunized with the T-dependent antigen (4-hydroxy-3-nitrophenyl)acetyl (NP) chicken γglobulin (CGG) to analyze specific antibody production. All animals were re-injected with the soluble antigen at day 34 to further study the secondary immune response. The detection of serum NP-specific antibodies showed only a slightly retarded and decreased primary response in both mutants, whereas class switch and affinity maturation occurred as in wt mice. In the secondary immune response virtually no difference to wt controls existed indicating a normal memory B cell generation and function. These results were completely unexpected, as conventional histology and immunohistochemistry with stainings for proliferation (mKi-67), germinal center B cells (peanut agglutinin), T-cell and dendritic cell markers of all major lymphoid tissues which were performed at three time points after primary immunization had revealed a completely resolved architecture of the lymphoid tissues without any initiation of classical germinal center reactions. In ultrastructural analysis the tight membrane adhesions (< 5 nm) between lymphocytes and dendritic cells observed in wt mice ($n = 6$) were missing in CD18 null mutants ($n = 6$) in all lymphoid tissue sections analyzed (lymph nodes, spleen, Peyer's patches, tonsils). However, rudimentary bridges of membrane contacts existed. We here provide further evidence that formerly described lymphoid structures and germinal center formation are not necessarily required to mount an antigen specific immune response, but that residual structures can compensate.

025

β1 Integrin-Mediated Interaction of T Cells and Extracellular Matrix Proteins (ECMP): Identification of Novel Inhibitors by Random Oligopeptide Phage Display Library Screening

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Our previous work suggested that integrin α chain-specific antagonists may be of limited benefit in anti-inflammatory therapy (Rao et al. J Immunol 165:4935–4940, 2000), as they inhibit the effects on cells of specific ECMP amongst a redundancy of such matrices. We have therefore used a phage display library expressing 3.7×10^9 independent cyclic 7-mer peptides (New England Biolabs) to identify novel, broad spectrum antagonists that recognise the common β1 integrin chain. A $CD8^+$ T cell line (10^6 cells) was incubated with 2×10^{11} phage, washed and specifically bound phage eluted with an excess (50 μg per mL) of the β1 chain-specific mAb, 4B4, which has potent, broad spectrum inhibitory effects on ECMP-mediated T cell activation. Supernatant phage were amplified in *E. coli* and subjected to two further rounds of binding, elution and amplification. Final supernatant was plated and 40 plaques isolated. Sequencing of variable DNA revealed a series of identical clones expressing cysteine-flanked cyclic peptides. Peptides expressed by the most abundant clones (Cys-PSSVRHS-Cys, 7 clones; and Cys-SLRPLEI-Cys, 5 clones) were synthesised, cyclized and > 95% purified. These peptides inhibited adhesion of $CD4^+$ T cells to type I collagen and fibronectin only weakly ($IC_{50} > 250$ μM), whereas their effects on T cell activation by anti-CD3 coimmobilised with collagen or fibronectin were more potent (IC_{50} approx 20 μM). We have thus identified peptides that inhibit β1 integrin-mediated signalling but may not influence cellular binding to ECMP and thus tissue integrity. These agents may be of therapeutic interest in psoriasis.

026

Isolation of Autoreactive T Helper 1 and 2 Clones by MACS Secretion Assay: A New and Sensitive Approach to Monitor T Cell Reactivity in Pemphigus

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Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are characterized by autoantibodies to the desmosomal glycoproteins desmoglein 3 (Dsg3) and desmoglein 1 (Dsg1), respectively. The relevance of autoantibodies is well-defined, but the cellular immune mechanisms involved in autoantibody formation are mostly unknown. In this study, peripheral Dsg-specific T cell clones of Th1 (IFN-c secretion) and Th2 type (IL-4 secretion) were cloned by MACS secretion assay. A total of 5 patients with PV and 2 patients with PF were studied. All patients showed IgG1 and IgG4 reactivity to Dsg1 (PF) or Dsg3 (PV). Patients with acute onset disease (PV = 1; PF = 1) had a Th1/Th2 cell ratio of 2.5 : 1. Patients with chronic active disease (PV = 2; PF = 1) had significantly more Th1 than Th2 cells (ratio 20 : 1) while patients in remission (PV = 2) had a Th1/Th2 cell ratio of 1 : 1. From each patient, 96 Th1 and 96 Th2 cell clones were generated after stimulation of PBMC with Dsg1/3 of whom 8–14% were responsive to Dsg1/3 (SI: 3–28; background: 1200 cpm). Upon *in vitro*-stimulation with Dsg1/3, all of the autoreactive Th1 and Th2 cells maintained their cytokine secretion pattern. *In vitro* responsiveness to Dsg1/3 of the autoreactive T cells declined over time: by the 8th re-stimulation, only 3% of the T cell clones proliferated in response to Dsg1 (SI: 3–4.5; background: 750 cpm) or Dsg3 (SI: 3–6; background: 750 cpm). Noteworthy, T cell clones of patients with chronic active or remittent disease and immunosuppressive treatment lost their proliferative capacity after the 2nd re-stimulation with Dsg1/3. These observations strongly suggest a bias towards the selection of distinct autoreactive Th1 and Th2 clones upon repeated *in vitro* stimulation with Dsg1/3. The MACS secretion assay allows for the screening of a wide spectrum of autoreactiveTh1 and Th2 cells which will facilitate the identification of the relevant immunodominant T cell epitopes of Dsg1 and Dsg3 and of the role that Th cells play in the pathogenesis of pemphigus.

027

Antibodies to BP180, But Not to BP230, Induce Subepidermal Blisters in Cryosections of Human Skin

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Bullous pemphigoid (BP) is a subepidermal autoimmune blistering disease associated with autoantibodies to the hemidesmosomal bullous pemphigoid antigens 180 (BP180) and 230 (BP230). Most sera from BP patients recognize epitopes clustered within the 16th noncollagenous (NC16A) domain of BP180. In a passive transfer mouse model of BP, rabbit antibodies against murine BP180 induce subepidermal blisters when injected into neonatal mice. In addition, using cryosections of human skin, patients'sera were previously shown to generate dermal-epidermal separation when coincubated with leukocytes and complement from healthy volunteers. However, the specificity of pathogenic autoantibodies found in the peripheral blood of BP patients has not yet been elucidated. In the present study, by the use of the cryosection model, we show that sera from all of 11 BP patients and from 2 rabbits, immunized against human BP180 NC16A, induced a dermal-epidermal separation. This finding was confirmed with the use of IgG purified from patients' sera, while sera and purified IgG from healthy controls or preimmune rabbits were not pathogenic. Interestingly, patients' autoantibodies affinity-purified against a recombinant form of BP180 NC16A, coupled to an agarose matrix, retained their blister-inducing capacity. In contrast, patients' IgG, that was depleted of reactivity to NC16A, lost this ability. In addition, patients' autoantibodies purified against a recombinant fragment of the C-terminus of BP180 as well as monoclonal and polyclonal antibodies to both intracellular and C-terminal sites of BP180 did not induce subepidermal splits in this model, neither did monoclonal antibodies to BP230. For the first time, we demonstrate the pathogenic relevance of autoantibodies to BP180 from patients with BP. Autoantigenic sites mediating this effect localize to the NC16A domain of human BP180. This experimental model should greatly facilitate the further dissection of the cellular and humoral pathogenesis of blister formation in human BP.

028

Intra-Epidermal Blister Formation in a Patient with Non-Herlitz Junctional Epidermolysis Bullosa who is Compound Heterozygous for COL17A1 Mutations

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Non-Herlitz junctional epidermolysis bullosa is a nonlethal variant of junctional epidermolysis bullosa with an autosomal recessive inheritance pattern. Pathogenic mutations have been found in the *COL17A1* gene encoding type XVII collagen, a homotrimeric, hemidesmosomal transmembrane protein. The ultrastructural plane of cleavage for type XVII collagen-deficient non-Herlitz JEB is consistently through the lamina lucida. In this study, we describe a child with type XVII collagen deficiency having an unusual intraepidermal level of blister formation, just above the plasma membrane. Immunofluorescence microscopy of nonlesional skin showed absence of the type XVII collagen endodomain and presence, although reduced, of the shed type XVII collagen ectodomain, synonymously to linear IgA disease antigen. Intact type XVII collagen was entirely nondetectable in lesional skin and in cultured keratinocytes of the patient. RT-PCR and sequence analysis of mRNA, in combination with DNA analysis, identified two novel mutations in *COL17A1*: a 3′-acceptor splice site mutation in intron 21 (COL17A1: 1877–2 A > C), and a single base pair deletion in exon 48 (COL17A1: 3432delT). This latter exon 48 mutation results in out-of-frame reading, thereby generating a premature termination codon, and in addition to in-frame exon 48 skipping at very low abundance. The splice site mutation in intron 21 results in skipping of exon 22 or to use of an alternative splice site in exon 22. Both events lead to in-frame transcripts that code for polypeptides with a small deletion in the 15th collagen (COL15) domain of type XVII collagen. The intraepidermal, nonjunctional, blister formation in this patient may be caused by different turnover rates between type XVII collagen in transmembrane position (high turnover) vs. shed type XVII collagen ectodomain in the lamina lucida (low turnover). We speculate that residual type XVII collagen ectodomain present in the lamina lucida has adhesive properties, which strengtens the lamina lucida, making the BP180-deficient hemidesmosomal plaque the locus minoris resistensiae in this patient.

029

Recombinant Analysis of Collagen XVII Shedding and Triple-Helix Formation

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Collagen XVII, a transmembrane hemidesmosomal component, is mutated in junctional epidermolysis bullosa and acts as an autoantigen in blistering skin diseases. It exists in two forms, as the full-length, triple-helical transmembrane molecule and as a 120-kDa cleaved ectodomain. The shedding occurs in the noncollagenous NC16a domain adjacent to the transmembrane region, but the exact cleavage site and the detailed mechanism are not known. Here an expression vector coding for the full-length collagen XVII was modified by three different deletions in the NC16a domain, and the mutated collagens were transiently expressed in COS-7 cells. The deletion of the hole NC16a domain inhibited the shedding of the ectodomain into the cell media, and also a minor deletion of amino acids 508–559 impeded the cleavage. In contrast, the deletion of the first 18 amino acids of the NC16a domain neither prevented the shedding nor reduced the amount the shed ectodomain. Trypsin digestions as probes for protein folding indicated that all three different deleted recombinant collagens could fold into a stable triple-helical conformation with melting temperatures over the body temperature. In summary, our results show that furin or furin-like proteinases are not the genuine convertase responsible for the release collagen XVII since the shedding was not inhibited by the deletion of the 18 first amino acids of NC16a domain containing the putative furin/PACE recognition sequence R-I-R-R. Interestingly, the triple-helix folding of collagen XVII does not require the presence of the first 18 amino acids of the NC16a domain which are homologous to a suggested association domain of collagen XIII, another transmembrane collagen.

030

Ultraviolet Irradiation Downregulates type II TGF-β Receptors Resulting in Inhibition of Connective Tissue Growth Factor-Dependent Type I Procollagen Gene Expression in Human Skin *In Vivo* and Human Skin Fibroblasts

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Reduced synthesis of type I procollagen (COL-I) contributes substantially to impaired dermal function in photoaged human skin. Transforming growth factor-β (TGF-β)/Smad pathway, directly and indirectly through induction of connective tissue growth factor (CTGF), regulates COL-I synthesis. We have investigated the mechanism by which UV irradiation impairs production of COL-I in human skin *in vivo* and cultured human skin fibroblasts (FBs). UV irradiation (2MED, UVB source) inhibited type II TGF-β receptor (59%), but not type I TGF-β receptor, CTGF (56%), and COL-I (52%) mRNA expression ($p < 0.05$, $n = 6$) 8 h post UV, as determined by real-time RT/PCR and *in situ* hybridization, in the dermis of human skin *in vivo*. Cultured human skin FBs constitutively expressed TGF-β1/2/3, CTGF, and COL-I mRNAs and proteins. Neutralizing pan-TGF-β antibody reduced CTGF and COL-I mRNA 58% and 79%, respectively ($p < 0.05$, $n = 3$), indicating that CTGF and COL-I expression are dependent on TGF-β. UV irradiation (30 mJ per cm^2, UVB source) of cultured human skin FBs resulted in a time dependent inhibition of CTGF (76%, $p < 0.05$, $n = 3$) and COL-I (76%, $p < 0.05$, $n = 3$) mRNA expression. UV reduced TGF-β-dependent binding of stimulatory transcription factors to TGF-β/Smad response elements in the CTGF and COL-I(á2) gene promoters. UV reduced type II, but not type I, TGF-β receptor mRNA and protein levels, and specific cell surface TGF binding 71%, within 8 h post UV ($p < 0.05$, $n = 4$). This down-regulation of TGF-β type II receptor resulted from reduced gene transcription. Overexpression of type II TGF-â receptor prevented UV induced reduction of COL-I gene expression. These data demonstrate that down-regulation of type II TGF-β receptors is responsible for UV reduction of TGF-β-regulated CTGF and COL-I gene expression in human skin FBs. Therefore, UV down-regulation of type II TGF-β receptors is a critical mediator of reduced COL-I synthesis in photoaging.

031

Gelatinolytic Activity in Melanoma Biopsies is Concentrated in Areas of Tumor–Stroma-Interactions and Does Not Colocalize with the Expression of Matrix Metalloproteinases

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The involvement of extracellular matrix degrading enzymes like matrix metalloproteinases and serine proteases during the process of tumor invasion and metastasis is well established. The progression of malignant melanoma has been correlated to the expression of MMP-2, and the *in vitro* invasiveness of melanoma cells is dependent on the activation of MMP-2 by MT1-MMP. To study the distribution of gelatinolytic activity on tissue sections we performed *in situ* zymography. This technique allows to localize areas where the balance of proteases and inhibitors is altered to favour the breakdown of extracellular matrix molecules. In 28/41 (68%) of primary tumors and 6/6 lymph node metastases considerable proteolysis was detected at the invading part of the tumor and within connective tissue septal structures inside the tumor. By microdissection and gelatin substrate zymography we could attribute this gelatinolytic activity to activated MMP-2. In general, gelatinolysis was concentrated at sites where melanoma cells had contact to the extracellular matrix, indicating an important role of tumor–stroma interactions for the regulation of proteolytic activity. These data are in agreement with the observation that melanoma cells *in vitro* activate MMP-2 only upon contact with native type I collagen. Next we investigated the expression and localization of MMP-2 and MT1-MMP in melanoma biopsies by immunohistochemistry. We investigated 41 primary melanomas and 6 lymph node metastases. In all cases both enzymes could be detected on the tumor cells as well as on adjacent connective tissue cells in variable amounts. In lymph node metastases MMP-staining was weaker compared to primary tumors. The detection of metalloproteinases like MMP-2 by immunohistochemical staining even in areas with no effective gelatinolytic activity strongly emphasizes the importance of more fuctional assays like *in situ* zymography when investigating balanced systems like proteases and their inhibitors. The presence of proteases alone is not necessarily linked to degradation of the corresponding substrates. The effective proteolytic capacity seems to be influenced by environmental factors like the extracellular matrix.

033

Adenovirus-Transduced DC Induce Broad but Mainly Virus-Specific Immune Responses in Human CD8$^+$ T Cells

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Genetic immunization through *ex vivo* transduction of dendritic cells (DC) with adenoviral vectors (Ad) has been suggested to be an effective approach to enhance antitumor immunity by activating a broad range of peptide-specific CD8$^+$ T cells. In our study, we wanted to analyze whether the *in vitro* priming of T cells with gp100-transduced, mature DC resulted in a more efficient induction of CTL when compared to peptide-pulsed DC. Transduction of DC using adenoviral vectors did not affect their phenotype and resulted in DC strongly expressing the melanoma antigen gp100 as assessed by antibody staining. T cells stimulated with Ad-transduced DC (Ad-DC) showed an earlier and stronger up-regulation of cytokines (IFN-γ, IL-2) after stimulation as compared with T cells stimulated with peptide-pulsed DC. Furthermore, T cells stimulated with Ad-DC showed reactivity against several gp100-epitopes, suggesting the simultaneous presentation of multiple peptide-epitopes in an immunogenic form. Surprisingly, gp100-specific T cell responses induced by Ad-DC decreased upon several restimulations while in parallel, Ad-virus-specific, MHC class-I restricted T cell responses were elicited. In comparison, only peptide-pulsed DC were able to induce long-lasting, melanoma antigen specific cytotoxic T cells. Thus, antiadenoviral T cell responses may provide a "helper" effect by inducing the release of high amounts of IL-2 and IFN-γ and therewith enhance CTL induction. In addition, priming with Ad-DC might help to identify the epitope specificity of CD8$^+$ T cells in individual melanoma patients and may be useful for defining relevant peptides for vaccination strategies in these patients. A prime/boost vaccination strategy with the initial induction of immune responses by Ad-DC and a boost with peptide-loaded DC at later stages should be evaluated based on these data.

035

Antigen-Specific, Poly Cation-Based Vaccines: Mode of Action

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Injection of tumor antigens together with poly aminoacids (e.g. poly arginine, poly lysine) has been shown to protect animals against tumor challenge. The goal of this study was to unravel the mechanism(s) underlying this phenomenon. For this purpose, we used βgalactosidase (βgal) as a surrogate antigen. DBA/2 mice were injected i.d. on days 0 and 14 with βgal complexed to poly arginine (thereafter referred to as protein vaccine (PV)) and inoculated on day 24 with βgal-expressing RENCA cells. PV treatment protected 8/8 animals against a challenge with RENCAlacz, but none of 5 against the parental RENCA cells. Antigen-specific protection was found in only 3/8 βgal recipients. None of the negative control animals (naive, pArg recipients) but 7/8 mice that had received pDNA coding for βgal (positive control) were able to reject the RENCAlacz inoculum. To test whether PV administration induces a βgal-specific immune response, animals received two i.d. injections and were then analyzed for specific B and T cells. As for the tumor protection assay, PV injection was superior to βgal administration in its ability to induce specific Abs as well as IFN-γ-producing T cells recognizing the immunodominant class I-restricted βgal epitope. *In vivo* T cell depletion studies demonstrated that the protective effect is critically dependent on CD8$^+$ but not on CD4$^+$ T cells. Specific Abs appeared not to contribute to the tumor-destructive events as they failed to stain intact RENCAlacz cells. Interestingly, priming of CD8$^+$ T lymphocytes required the presence of CD4$^+$ cells when mice were immunized with βgal pDNA but not when using the PV. To learn more about the fate of the PV at the injection sites, these were removed at defined time points (1.5 h, 1 and 11 d after injection) and thus treated animals were analyzed for βgal-specific immunity on day 14. Results obtained showed that removal of the i-site as early as 1.5 h after PV application does not affect the elicitation of βgal-specific T and B cells. Evaluation of the draining lymph nodes revealed the presence of βgal protein already by 1.5 h after its i.d. injection. Cells displaying the immunodominant, class I-restricted peptide epitope were first detected 24 h after PV treatment followed by the appearance of antigen-specific T cells on day 5. Together, these results extend our knowledge on the immunostimulatory properties of poly cations by demonstrating that their coadministration with βgal triggers a protective and specific CD8 T cell response independent of (i) CD4$^+$ T helper lymphocytes and (i) the prolonged presence of the i-site. It remains to be seen whether this is due to the ability of poly cations to act on the T cells themselves, the antigen presenting cells involved or both.

032

Invasion of Melanoma Cells into Dermal Extracellular Matrix *In Vitro*: Evidence for a Role of Cysteine Proteases in Matrix Degradation

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Local invasion of melanoma cells into the dermal connective tissue is the first step in the complex process of metastasis. Recently, we have developed an improved skin organ culture model that allows the detailed study of melanoma cell invasion *in vitro*. In this model high (BLM) or low (530) invasive melanoma cells were seeded on the dermal side of dead deepidermized dermis and the composits were cultured for 14 d at the air/liquid interface. The high invasive cells rapidly invade the tissue, whereas the low invasive cells do not enter the tissue. As it is well known that proteolytic alteration of the extracellular matrix (ECM) is a key event in tumor cell invasion, we investigated the expression and enzyme activity of different proteases. Immunohistochemistry of this *in vitro* grown melanoma reveals the expression of different members of the matrix metalloproteinase (MMP) family as well as urokinase plasminogen activator (uPA) only by the BLM cells and the cysteine protease cathepsin B in both cell lines. To analyze the enzymatic activity of these different proteases in the process of ECM degradation, we performed in-situ gelatin zymography of cryosections under different environmental conditions. Areas of gelatinolysis were only detected in composits cultured with the high invasive BLM cells. Interestingly, in-situ zymograms incubated with an acidic buffer, which favours cysteine protease activity show much more gelatinolysis compared with zymograms incubated with a neutral buffer which favours neutral protease activity. Furthermore, the gelatinolytic activity at acidic conditions is inhibited by E-64 and Leupeptin, both inhibitors of cysteine proteases, but not by EDTA, an inhibitor of MMPs. As cysteine proteases like cathepsin B are capable of degrading different components of the extracellular matrix, these results indicate a major role for acidic proteases for the invasive process of melanoma cells *in vitro*.

034

PolyG-Oligodinucleotides Containing CpG Motifs have no Beneficial Impact on the Induction of Antitumoral Cytotoxicity in CD8-T-Cells when used in a Therapeutic Model

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CpG ODNs using flanking polyG sequences have been reported to act preferentially on the proliferation of CD8-T-cells by transmitting a signal through the formation of hexameric structures and subsequent activation of scavenger receptors. We have tested the capacity of the polyG-CpG-ODN1628 to induce proliferation of antitumoral CD8 cells in the well described P815 mouse mastocytoma tumor model. For this purpose animals were challenged with the tumor and subsequently injected with 30 µg ODN 1628 or a control oligo in the tumor area for 12 days. Neither tumor growth nor survival time showed differences between the two groups. When looking for the quantitative induction of tumor-specific CD8 cells against the two dominant tumor epitopes (P1A and P1E) by tetramer staining (Kd-P1E and Ld-P1A) and by CDR3 spectratyping which is able to identify the magnitude of the public response against P1A and P1E (Vb1-Jb1.2 and Vb1-Jb2.5) we found similar induction in both groups. To exclude differences in the induction of cytotoxicity we tested purified T-cells from the draining lymph nodes and the spleen of the injected animals in a Cr51-release assay using labeled P815 as targets. Additionally we sorted antitumor-specific CD8 cells by FACS sorting and analyzed this population in the two groups for cytotoxicity associated molecules including granzyme B, perforine, IFNγ and FasL by quantitative Taqman assisted RT-PCR. Again, no differences in direct cytotoxicity or in the expression of cytotoxicity associated molecules were detected in the two groups. In order to screen for the described *in vitro* activity of ODN 1628 we used a model for the activation of antigen specific CD8 cells by preparing CD8 cells from a TCR tg mouse in which all CD8 cells use the T-cell receptor specific for the LCMV antigen gp 33. Using these cells in the appropriate antigen stimulating conditions we found a clear increase in the proliferation rate of cells stimulated in the presence of ODN 1628 as compared to the control. However, when the activated cells were sorted again (as determined by the expression of CD 69), we found no differences in the transcription of cytotoxicity associated molecules as determined by quantitative Taqman PCR. Thus, although capable of inducing CD8 cell proliferation *in vitro*, polyG-CpG-ODNs are not suitable to enhance antitumor specific CD8 cell response *in vivo* in our model.

036

IL-1α and IL-1ra are Homeostatically Regulated in Murine Keratinocytes *In Vitro* and *In Vivo*

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Interleukin (IL)-1α is a potent proinflammatory cytokine constitutively expressed by keratinocytes and comprises one member of an expanding gene family including IL-1β, IL-18 and the specific inhibitory molecule, IL-1 receptor antagonist (IL-1ra). Although homeostatic regulation of this system has been suspected, there is currently little evidence for this. PAM 212 keratinocytes were exposed to increasing concentrations of IL-1α or IL-1ra for 24 h and ELISA performed to assess IL-1ra or IL-1α production, respectively. In this system, IL-1ra release was induced in a dose-dependent manner following addition of mIL-1α from 105 ± 38 pg per mL (unstimulated) to 2185 ± 104 pg per mL (10 ng per mL IL-1α, $n = 5$ experiments). Similarly, treatment of keratinocytes with IL-1ra resulted in increased IL-1α release in a dose dependent fashion with maximal IL-1α release (1043 ± 57 pg per mL) at 1 ng per mL IL-1ra ($n = 3$). In each case, addition of a blocking anti-IL-1 receptor type 1 (IL-1R1) antibody inhibited release of the induced cytokine, indicating that the feedback loop involved IL-1R1. Supernatant LDH content was not increased by keratinocyte stimulation excluding cell death as a cause of the cytokine release. To determine whether a similar phenomenon occurred *in vivo* we examined cytokine release by epidermal sheets derived from previously described transgenic mice in which over-expression of IL-1α or IL-1ra was targeted to basal keratinocytes. In concordance with our *in vitro* findings, epidermal sheets from IL-1α transgenic mice released increased IL-1ra (5006 ± 140 pg per mL) compared with wildtype animals (611 ± 18 pg per mL, $n = 3$) and this increase was specifically inhibited by the presence of an anti-IL-1R1 antibody. Conversely, increased IL-1α release was observed in two independent IL-1ra transgenic lines (678 ± 35 pg per mL and 530 ± 121 pg per mL) compared with wildtype mice (164 ± 29 pg per mL) and this increase was also reduced to wildtype levels by antibody blockade. These findings provide the first evidence for the presence in keratinocytes of a homeostatic mechanism mediated via the type-1 IL-1 receptor in which production of IL-1 agonists and antagonists are mutually interdependent. Our findings provide an explanation for the cutaneous inflammatory response to IL-1ra previously observed in clinical trials and underscore the complexity of the keratinocyte cytokine network.

037

UVB-Induced Skin Inflammation in Mice is Mast Cell-Dependent

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We have recently shown that endothelin-1 (ET-1) induces pronounced inflammatory responses in murine skin and that ET-1-induced skin inflammation is MC-dependent. Here, we have studied the role of mast cells (MC) in UVB-induced dermatitis, a cutaneous inflammatory reaction characterized by immediate and strong up-regulation of ET-1 expression by epidermal keratinocytes. Balb/c mice were used in extensive pilot experiments to characterize cutaneous inflammation after irradiation of ears with UVB in nonpigmented murine skin. UVB (500 mJ per cm^2) induced a robust inflammatory reaction in Balb/c mice, as assessed by repeated measurements of ear swelling (90 ± 12 μm, at 18 h). Ear skin of genetically MC-deficient Kit^W/Kit^{W-v}-mice that is also not pigmented exhibited significantly reduced swelling responses after UVB irradiation (24.1 ± 5.1 μm at 18 h, $p < 0.001$). To test whether the adoptive transfer of MC to MC-deficient mice would restore impaired inflammatory responses to UVB, we irradiated Kit^W/Kit^{W-v}-mice reconstituted with bone marrow-derived cultured MC (left ears, Kit^W/Kit^{W-v} + BMCMCs) or vehicle (right ears, Kit^W/Kit^{W-v} + Veh). Earswelling in MC-reconstituted ears after UVB irradiation was significantly increased as compared to UVB-treated control ears (Kit^W/Kit^{W-v} + BMCMCs: 78.7 ± 11.9 μm vs. Kit^W/Kit^{W-v} + Veh: 24.1 ± 5.1 μm at 18 h, $p < 0.005$), indicating that UVB-induced inflammation in murine skin is, at least in part, MC-dependent. Since UVB irradiation rapidly up-regulates expression of ET-1, one of the most potent MC-secretagogues, we hypothesize that ET-1-mediated activation of dermal MC contributes to UVB-induced cutaneous inflammation.

038

Epidermal Overexpression of Stratum Corneum Chymotryptic Enzyme in Mice; a Model for Chronic Itchy Dermatitis

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The serine protease stratum corneum chymotryptic enzyme (SCCE) is believed to play a role in the desquamation process. Excessive expression of SCCE was recently shown in the epidermis of psoriatic patients and we have also found SCCE overexpressed in chronic lesions of atopic dermatitis. To further elucidate the possible involvement of SCCE in skin pathology, we produced transgenic mice that overexpressed human *scce* mRNA under a viral promoter. Phenotypic changes were only found in skin. The transgenic mice expressed human SCCE in suprabasal epidermal keratinocytes and furthermore, developed pathological skin changes similar to those seen in inflammatory skin diseases in humans, such as increased epidermal thickness, hyperkeratosis and dermal inflammation. In addition, these mice also showed signs of severe itching. Our results suggest that SCCE may be involved in the pathophysiology of inflammatory skin diseases and is therefore a potential target for tissue-specific treatments.

039

Induction of an Intralesional Th2 Phenotype and Regression of Psoriasis During Interleukin 4 Therapy of Humans

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The inflammation leading to psoriasis is dominated by interferon-γ producing tpye 1 T cells. Immune deviation of type 1 into interleukin-4 (IL-4) dominated type 2 responses improves inflammatory autoimmune diseases in mice. Since IL-4 efficiently induces IL-4 in human T cells, we investigated safety and efficiency of systemic IL-4 application in the treatment of severe psoriasis in a dose escalation study. Five groups, each including 4 patients, were attributed to increasing doses of IL-4. The IL-4 was self-injected over a period of 6 weeks. The follow up included weekly PASI documentation, skin biopsy and analysis of a series of immune parameters that potentially can be affected by IL-4. A total of 22 patients was recruited, 20 terminated the study, two interrupted. Only in one patient side-effects > grade I were observed. In 18/20 patients the PASI decreased by 60–80% within six weeks and no rebound occured during the 6 weeks of follow up. Mononuclear cells including T cells, CD4/CD8-ratio and neutrophil counts remained unchanged. The number of eosinophilic granulocytes increased significantly, while the number of B cells decreased slightly. Upon direct *ex vivo* analysis $CD4^+$ and $CD8^+$ T cells produced similar amounts of IL-2, TNF-α or IFN-γ before and during therapy. In sharp contrast, a small (± 2%) but distinct population of IL-4 producing $CD4^+$ T cells was induced during therapy. No IL-4 producing $CD8^+$ T cells were detected. Induction of IL-4^+ $CD4^+$ T cells was associated with a strong reduction in $CCR5^+$ T cells and a reduction of the IFN-γ/IL-4 ratio up to 100 fold, as determined by quantitative real time PCR, in the psoriatic lesions. Thus, the significant improvement of psoriasis during IL-4 therapy was associated with a strong shift of a Th1 towards a Th2 phenotype. Since IL-4 was, in addition, well tolerated, IL-4 induced immune deviation may be a promising therapy for human inflammatory autoimmune diseases.

040

TNF- and IL-1 Signal Transduction Pathways are Crucially Involved in Wound Healing

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Previously, it has been shown that the inflammatory cytokines TNF and IL-1 are activated in wounds (Lit.). TNF and IL-1 regulate proliferation, differentiation and apoptosis. TNF and IL-1 signal transduction involves TNF receptor p55 (TNF-R55), IL-1 receptor I (IL-1-RI), acid and neutral sphingomyelinase (A- and N-SMase) and the TNF-R55 adapter protein FAN, important for the activation of N-SMase. We explored the function of this signaling pathways in wild type and in transgenic mice. Full thickness skin wounds of 1.5 cm diameter were induced and healing was examined after photography and computer-assistant evaluation. In TNF-R55 deficient mice we found a significant delay in wound healing related to a reduced reepithelization. A severe delay in the healing process occurred in TNF-R55/IL-1 double decient animals. In these animals not only reepithelization, but also wound contraction were nearly absent. In wild type mice an early increase in epidermal proliferation during wound healing was found. The increase in proliferation was reduced in FAN cytokine receptor deficient mice. A late increase in A- and N-SMase activity during epidermal differentiation occurred in wild type, but not in deficient mice. In FAN deficient mice we found a reduction in reepithelization showing the importance of N-SMase for this process. In summary, we showed that TNF and IL-1 signal transduction including TNF-R55, the adapter protein FAN, IL-1-RI, and A- and N-SMase are important for wound healing.

041

Caspase-14 But Not Caspase-3 and Caspase-8 Expression by Epidermal Keratinocytes is Regulated by Retinoids in a Differentiation-Associated Manner

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Caspase-14 is the only member of the caspase family that shows a restricted tissue expression. It is mainly confined to epidermal keratinocytes (KC) and in contrast to other caspases, is not activated during apoptosis induced by UV-irradiation or cytotoxic substances. Since it is cleaved under conditions leading to terminal differentiation of KC we suggested that caspase-14 plays a role in the physiological cell death of KC leading to skin barrier formation. Here we show that retinoic acid (RA), at concentrations inhibiting terminal differentiation of KC, strongly suppressed caspase-14 mRNA and protein expression by KC in monolayer culture and in a 3-dimensional *in vitro* model of differentiating human epidermis (skin equivalent, SE). By contrast, the expression of the caspases 3 and 8, which are both activated during conventional apoptosis, was increased and unchanged, respectively, after RA treatment. In addition to inhibitio of differentiation in SE, RA treatment lead to KC apoptosis and activation of caspase-3, which was undetectable in differentiated control SE. Since this occurred in the absence of detectable caspase-14, our data demonstrate that caspase-14 is dispensible for KC apoptosis. The fact that in contrast to caspase-3 and caspase-8, caspase-14 is regulated by retinoids comparable to other KC differentiation-associated proteins, strongly suggests that this caspase, but not caspase-3 and –8, plays a role in terminal KC differentiation and skin barrier formation.

042

The Antiapoptotic bcl-2 Proteins are Highest and Caspases are Not Activated in Basal Keratinocytes Expressing High Levels of p63

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A keratinocyte population enriched in stem cells, expressing highest levels of β1-integrin, is protected from ''anoikis'' (apoptosis from loss of adhesion). The aim was to evaluate the intracellular mechanisms involved in keratinocyte ''anoikis''. To this purpose, we isolated three populations of keratinocytes, based on their adhesive properties to type IV collagen: rapidly adhering cells (pop 1), overnight adhering cells (pop 2), and not adhering cells (pop 3). Apoptosis was striking in cultured epidermis reconstructed on de-epidermized dermis (RE-DED) from pop 3, nearly absent in RE-DED from pop 1 and intermediate in RE-DED from pop 2, as shown by TUNEL. p63 transcription factor, a stem cell marker with antiapoptotic functions, was more expressed in pop 1 than in pop 2, and nearly absent in pop 3 from cultured keratinocytes, as measured by Western blot. Caspase-8 and Bid were processed in pop 2 and 3, but not in pop 1. Bax and Bad protein expression was high in pop 3, decreased in pop 2, and very low or nearly absent in pop 1. On the other hand, Bcl-2, Bcl-xL and Mcl1 levels were markedly higher in pop 1 than in pop 2, and were absent in pop 3. Caspase-9 and – 6 were activated in pop 3 and, to a lesser extent, in pop 2, while they were not activated in pop 1. Caspase-3 and PARP were cleaved only in pop 3. The IAP protein survivin was more expressed in pop 1 than in pop 2, while the cleaved fragment of hILP/XIAP was higher in pop 3 than in pop 2, and absent in pop 1. These results confirm that keratinocytes enriched in stem cells are protected from ''anoikis''. In addition, this work shed light on the intracellular events during ''anoikis''.

043

Stat3 in Epidermal Keratinocytes Functions as UV-Sensor

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We investigated the antiapoptotic role of epidermal Stat3 using keratinocyte-specific Stat3-disrupted mice, in which floxed Stat3 is ablated by Cre recombinase under the control of the keratin 5 promoter. Upon UVB irradiation, they exhibited an increased number of apoptotic keratinocytes, which was efficiently reversed by topical application of Stat3 gene. These results indicate that Stat3 in keratinocytes functions as an antiapoptotic regulator, so that keratinocytes survive against UV stress. We next examined the UVB effect on Stat3 in normal keratinocytes. UVB irradiation induced an early disappearance of tyrosyl-phosphorylated Stat3, followed by down-regulation of mRNAs of Bcl-xL and Stat3. Finally, Stat3 and Bcl-xL proteins disappeared on Western blotting 24 h after the irradiation. Interestingly, topical application of Stat3 gene rescued normal keratinocytes from UV-induced apoptosis as well as Stat3-disrupted keratinocytes. These results suggest that Stat3 firstly senses UVB to lose its transcriptional activity, leading to down-regulation of gene expression of Stat3 itself and antiapoptotic molecules such as Bcl-xL at its downstream. We conclude that Stat3 in keratinocytes dictates the fate of keratinocytes in two opposite ways; survival or apoptosis which may help to reduce the future oncogenic potential.

044

The p63 Serves as a Regulator of Keratinocyte Progenitor Ability During Wound Epithelialization

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The p63 protein was recently identified is a p53 homologue. Several isoforms of p63 lacking the acidic N terminus are expressed in the epidermis. These isoforms denominated as ΔNp63 are known to play a role in maintaining the proliferative potential of keratinocytes and preventing their terminal differentiation. More recently the p63 protein was found to identify the keratinocyte stem cell or progenitor cell population. The purpose of the study was to evaluate whether the p63 protein plays a role during wound epithelialization. For this purpose serial biopsies ($n = 32$) of human healing wounds taken 1–28 days after the injury were analyzed with the use of immunohistochemistry. The ΔNp63 expression in keratinocytes was compared with a cellular proliferation marker expression – a Ki67 protein. The ΔNp63 expression in the keratinocytes of migrating epithelial tongue was found decreased dramatically from the 1st day of epithelialization. At the same time, cells in the proliferating wound border, as revealed by Ki67 expression, were positively stained for p63 in the basal and several suprabasal layers. Similarly a few basal cells in the migrating epidermal tongue showed the p63 expression despite the fact that the majority of cells in this area were p63 negative. We speculate that down-regulation of ΔNp63 expression in the keratinocytes of migrating epidermal tongue enable these cells to leave out the progenitor compartment and to differentiate into a migratory phenotype. Up-regulated protein expression in the proliferating wound border may be necessary to prevent cells differentiation and to support their proliferative ability indispensable to yield new cells for epithelialization. Similarly scattered basal cells positive for ΔNp63 in the migrating epidermis, may be prerequisite for progenitor cell potential transfer and its maintenance in the restored epidermis covering the scar.

045

Activation of p38 Mitogen-Activated Protein Kinase Enhances Matrix Metalloproteinase-1 and –3 Expression by Stabilization of mRNA in Dermal Fibroblasts

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We have examined the role of p38 mitogen-activated protein kinase (MAPK) signaling pathway in the regulation of collagenase-1 (matrix metalloproteinase-1, MMP-1) and stromelysin-1 (MMP-3) expression in normal human skin fibroblasts. Tumor necrosis factor-α (TNF-α) activated extracellular signal-regulated kinase (ERK)1,2, Jun – N-terminal kinase (JNK), and p38 MAPK in dermal fibroblasts. TNF-α-elicited induction of MMP-1 and MMP-3 mRNA was potently inhibited by specific p38 MAPK inhibitor SB203580, whereas blocking the ERK1,2 pathway (Raf/MEK1,2/ERK1,2) by MEK1,2 activation inhibitor PD98059 had no effect under similar circumstances. Adenoviral gene delivery of constitutively active MKK3b together with wild type p38α greatly enhanced the production of proMMP-1 and proMMP-3. The enhancement of MMP-1 mRNA expression was 8.2-fold and that of MMP-3 1.9-fold. The increase in MMP-1 and MMP-3 mRNAs was associated with a delayed decay rate, because infection with an adenovirus for constitutively active MKK3b together with wild type p38α increased the half-life of MMP-1 mRNA from 2.3 h to 59 h, and the half-life of MMP-3 mRNA from 2.2 h to 48 h, as compared to control-infected cells. These results identify a novel mechanism by which MKK3b/p38α signaling pathway regulates MMP-1 and MMP-3 expression in fibroblasts.

046

RNase 7, a Novel Broadspectrum Antimicrobial Protein from Healthy Skin

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Human skin is highly resistant against the wide variety of potential harmful microorganisms to which it is constantly exposed. The basis for this natural resistance is not fully understood but one reason might be the release of antimicrobial proteins. We previously proved this hypothesis by the isolation of the Gram-negative bacteria killing peptide antibiotic human β-defensin-2 and very recently by the isolation of the broadspectrum peptide antibiotic human β-defensin-3. Since these two peptides were highly induced at sites of inflammation (i.e. in psoriatic lesions), but nearly absent in normal skin, we addressed the question, whether healthy skin contains antimicrobial peptides, especially those with activity against Gram-positive bacteria like *St. aureus*. To answer this question we analysed heel callus extracts for the presence of *St. aureus* killing activity and found high antimicrobial activity. This activity was purified using Heparin-sepharose chromatography followed by reversed phase and cation-exchange-HPLC. This led to the isolation of a highly cationic, antimicrobial protein (20 kDa by SDS-PAGE). N-terminal amino acid sequence analysis revealed a novel sequence with structural similarity to members of the RNase A superfamily, such as eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). Therefore we termed this novel antimicrobial protein RNase 7. Cloning the corresponding cDNA revealed keratinocytes as a cellular source. Interestingly RNase 7 gene expression was found to be induced by proinflammatory cytokines and bacteria. RNase 7 hydrolysed t-RNA indicating enzymatic RNase activity and killed *Staphylococcus aureus* and several other skin-relevant microorganisms at micromolar concentrations. Therefore we conclude that RNase 7 might participate in a first-line chemical defense system of healthy normal skin and might help to protect human skin from infections.

047

Characterization of Three Novel Candidate Genes in the MHC Region with Regard to Psoriasis Susceptibility

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Psoriasis is a common skin disease that affects 2–3% of the Caucasian population. The psoriasis susceptibility locus PSORS1 on chromosome 6p21 is now of a sufficiently small size to allow comprehensive in silico cloning of putative candidate genes. We have identified three novel candidates in this area (Candidate In Psoriasis Susceptibility 1–3, CIPS1-CIPS3). Both CIPS2 and CIPS3 genes are located telomeric to the HCR gene while CIPS1 lies centromeric. We amplified all 11 exons of these three genes in 10 PCR fragments and sequenced each from both ends in a series of Swedish psoriasis individuals, while comparing them to healthy population matched controls. Assembly of resulting sequences was performed in the Staden sequence assembly package and polymorphisms were identified using the Trace-Diff function. Using this association analysis we have found significant association to psoriasis by SNPs in all three genes. Further stratification reveals that the association of CIPS1 and CIPS2 can be explained by linkage disequilibrium with Cw*0602. Furthermore, the strongest associated SNP in CIPS3 is located in an exon which appears to be untranslated and is absent in an alternative splice variant. The role of this SNP in psoriasis and the possible biological significance of CIPS3 splice variants requires further study. With no remaining psoriasis candidates genes, we are left with the intriguing possibility of a synergistic effect of multiple genes or the reexamination of the role of HLA-C in psoriasis.

048

Nuclear Translocation of NF-IL6 (C/EBPβ) is Associated with Induction of Keratin 6 in Abnormal Epidermis

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It is established that keratin mutations cause genodermatoses. However, genotype-phenotype relationships remain unclear and how mutations cause the observed pathology is not understood. We have demonstrated that epidermolysis associated with genodermatoses is accompanied by increased expression of inducible keratins (K6, K16 and K17). These keratins are induced in other hyperproliferative states, absent in normal epidermis and constitutively expressed in skin appendages (hair follicle, sebaceous gland, and sweat gland). Their transcriptional regulation is not fully understood and it is not clear how tissue-specific gene regulation occurs. While studying transcription factors in normal and diseased epidermis (psoriasis, genodermatoses and wound healing), we have found that NF-IL6 is intimately connected with K6 and K16 gene expression. We have examined the control of K6 expression *in vivo* and find that translocation of NFIL-6 to the nucleus of suprabasal cells is always coincident with K6 expression. Frozen skin biopsies from normal subjects (4) and patients (8) with psoriasis or genodermatoses (EH, IBS, EPPK with defined mutations) were cryostat sectioned and treated with antibodies to K6, NFIL-6 or both. They were visualised with streptavidin-FITC or antibody conjugated Texas Red. Sections were examined by fluorescence photomicroscopy and confocal microscopy. Control sections with no primary antisera were prepared and some sections were incubated with propidium iodide (nuclear specific stain). NFIL-6 was cytoplasmic in basal cells, nuclear in suprabasal cells and absent from cornifying granular cells. Nuclear NFIL-6 was present in mid-epidermis of normal skin, dramatically increased in suprabasal cells of psoriatic epidermis and variably increased in the genodermatoses examined. Nuclear translocation of NFIL-6 was always accompanied by K6 expression in abnormal but not in normal epidermis. Thus, NFIL-6 is necessary but not sufficient for induction of K6 gene expression and other factors must be involved in this complex process.

049

Genetic Immunization of Mice with a Fusion Protein Between EGFP and Murine TRP2 Circumvents T Cell Tolerance and Mediates Protective Immunity to Melanoma

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We previously showed that genetic immunization of C57BL/6 mice with cDNA encoding human but not murine TRP2 was able to induce cellular and humoral immune responses to murine TRP2 associated with protection against metastatic growth of B16 melanoma. In the present study we investigated whether murine TRP2 could be rendered immunogenic when fused in frame to the fluorescent foreign protein EGFP. Using PCR techniques we constructed a cDNA encoding EGFP followed by aa30–517 of murine TRP2. Expression of both EGFP and murine TRP2 could be confirmed by immunocytochemistry and fluorescence microscopy in transiently transfected 293 cells. Importantly, genetic immunization of mice with EGFP-mTRP2 using the gene gun induced TRP2-reactive $CD8^+$ T cells associated with coat depigmentation as a sign of autoimmune-mediated destruction of melanocytes. Furthermore, immunization with recombinant adenovirus encoding EGFP-mTRP2 provided significant protection against metastatic growth of B16 melanoma. These results provide important insights for the development of antigen-specific immunotherapy of melanoma.

050

Molecular Genetic Analysis in a Large Group of Families and Sporadic Cases Affected by Incontinentia Pigmenti: Genomic Rearrangement, New Mutations and Genotype-Phenotype Correlations

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Incontinentia Pigmenti (IP) is an uncommon genetic skin disease, inherited as an X-linked dominant trait, carachterized by four classic cutaneous stages, perinatal inflammatory vescicles,-verrucous patches, a distinctive linear-reticular hyperpigmented pattern and a terminal atrophic stage. The disease is usually lethal in males and in the affected females IP causes highly variable abnormalities of the skin and adnexa, teeth, eyes and central nervous system. The gene responsible for IP has been found near the factorVIII locus in Xq28 and is the gene for NF-κB essential modulator (NEMO) that is required for the activation of the transcription factor NF-κB and is relevant for many inflammatory, immune and apoptotic pathways. The molecular genetic studies were performed in 12 families (28 subjects) affected by classic IP and in 6 sporadic cases, including two males, analysing genomic DNA from the proband, the parents and the maternal grandmothers, with an age ranging from six months to 70 years. The results confirm that genomic rerrangement in NEMO gene, namely large deletion of part of the gene within intron 3 and 3′ of exon 10, is the most frequent genetic abnormalities in IP patients, accounting for 18 out of 24 cases (8 families out of 12) and for 1 of the 6 sporadic cases. These deletions are absent in the two male cases and in one female in which mutation analysis of NEMO gene has been undertaken. In 1 sporadic case and in 1 family two new point mutation in the sequence of NEMO gene has been found. Interestingly in one of the two males a preliminary biochemical study revealed abnormalities in the pattern of cytokines related to immune modulation and apoptosis. The finding of genetic abnormalities of the NEMO gene in probands with classic four-stage disease, correlates strictly in mothers and grandmothers with the presence or absence of any sign of the disease, allowing us to define familiar and sporadic cases of IP, following the origin of the disease in all considered families.

051

Smad7 Binds the Transcriptional Coactivator p300 and Represses the Expression of Extracellular Matrix Genes in Dermal Fibroblasts

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Smad7 is an inhibitory Smad which acts as a negative regulator of transforming growth factor-β (TGF-β) signaling. Smad7 stably interacts with activated TGF-β type I receptor and interferes with the phosphorylation of receptor-regulated Smads. Recently it has been shown that Smad7 can be located in the nucleus, suggesting a direct role on gene transcription. To explore potential functions of Smad7 in the nucleus, we ectopically expressed Smad7 in dermal fibroblasts, together with various promoter/reporter gene constructs. In addition to blocking TGF-β-mediated Smad-specific gene transcription, Smad7 was also found to directly inhibit the acitvity of the minimal SV40 promoter, which contains a TATA box. Similarly, Smad7 was found to repress the activity of the human COL1A2 promoter, which also contains a TATA box. The latter does not seem to play a role in mediating Smad7 effect, as the activity of the human COL7A1 promoter, which is TATA box less, was also repressed by ectopic expression of full length Smad7. Smad7-driven repression of the minimal SV40 promoter could be antagonized by cotransfecting the transcriptional coactivator p300. Using a mammalian two-hybrid system we identified a direct interaction between Smad7 and p300. Using various deletion constructs for p300, we identified the region between amino acids 1732–2414 as binding Smad7. This region corresponds to the CH3 domain of p300, which is capable of binding members of the basal transcription machinery such as the TATA-box-binding protein (TBP), and TFIIB. No interaction of Smad7 with TBP was observed in a mammalian two-hybrid system. We propose a model in which Smad7 binds the CH3 domain of p300, thereby preventing the binding of TBP to p300 and subsequent gene transactivation. This mechanism may be responsible for the inhibitory effect of Smad7 on some extracellular matrix genes in dermal fibroblasts.

052

Effects of Mutations in ATP2A2 on Calcium Transport Across Sarco/Endoplasmic Reticulum (ER) Membrane

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ATP2A2 has been identified as the causative gene for Darier's disease (DD). This gene encodes the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2b) pump, which transports Ca^{2+} from the cytosol into the ER. This study aims to investigate the effects of *ATP2A2* mutations identified in DD patients on SERCA2 function. Several missense mutations have been engineered into *ATP2A2* full-length cDNA clones by site-directed mutagenesis. The wild type and the mutants were transiently over expressed in COS-1 cells. Microsomes were then isolated from these cells using a lysis/ultracentrifugation procedure. To determine the localisation and the expression level of mutant proteins in comparison with the wild type SERCA2b, immunodetection analysis including immunocytochemistry on intact cells and Western blotting of microsomal protein was performed using a SERCA2b specific antibody. The function of the SERCA2b pump was assessed by an oxalate-stimulated Ca^{2+} transport method. Our results have shown that mutations in *ATP2A2* impair Ca^{2+} transport activity of the SERCA2b pump. These results provide evidence for functional alteration of SERCA2 mutants in DD and improve our knowledge of the structural and functional relationship in SERCA2.

053

K6irs; a New Cytokeratin in the Inner Root Sheath of the Human Hair Follicle

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Analysis of the EMBO/Genebank database in an attempt to identify a human ortholog of a recently described cytokeratin, mK6irs, found in the inner root sheath of the mouse hair follicle, lead to the identification of a ca 80 kb Bacterial Artificial Chromosome (BAC) clone harboring six type II cytokeratin genes and one keratin pseudogene. One end of this clone overlapped the recently described type II hair keratin gene cluster. The genes found included human K6irs, K5, K6a, a gene similar to K6e, K6b, K6hf, and the pseudogene ψhHbA. Analysis of a human scalp cDNA library lead to the discovery of a full length K6irs cDNA transcript. Two dimensional separation of hair follicle keratin extracts and Western blot analysis showed the presence of multiple K6irs isoelectric variants present in the same region as that of the known K6 cytokeratins. *In situ* hybridization, indirect immunofluorescence microscopy as well as electron microscopy showed an exclusive expression of K6irs in the inner root sheath of the hair follicle. This expression initiated in the hair cell matrix region and showed a partially staggered expression in all three layers (Henle-, Huxley-, and cuticule layer) of the inner root sheath.

054

Identification of Binding Partners of the 72 kDa Heat Shock Protein in Human Keratinocytes by Two-Hybrid Analysis

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The 72 kDa heat shock protein (hsp72) is a stress inducible molecular chaperone that is involved in the protection from proteotoxic stress. In contrast to other cells and tissues human keratinocytes *in situ* and in culture express high levels of hsp72 without prior stress exposure. To identify proteins that interact with hsp72 in human keratinocytes we employed a yeast two-hybrid system (Matchmaker 2, Clontech). The yeast strain PJ69–4a was cotransformed with a keratinocyte-derived cDNA library (the "prey") and the complete coding sequence of human hsp72 (hsp70a, the "bait"), cloned into the appropriate yeast expression vectors. Bait–prey interactions were detected by reporter gene activation and confirmed by back-transformation. The interacting proteins were identified by cDNA isolation, sequencing, and GenBank comparison. The two-hybrid screen revealed interaction of hsp72 with SP100 (nuclear dot protein), thrombospondin, EGF-containing fibulin-like extracellular matrix protein (EFEMP1), and expressed sequence tags represented in GenBank. To further confirm the expression and colocalization of SP100 and hsp72 in human keratinocytes *in situ* we used immunofluorescence and confocal laser scan microscopy. SP100 is expressed in the nuclei of keratinocytes in culture and *in situ* ("nuclear dots"). Double staining revealed colocalization of hsp72 and SP100 in some of these dots in unstressed and heat treated HNK, and in normal human skin *in situ*. No colocalization could be detected in the squamous cell carcinoma cell line A431. These results for the first time describe the specific interaction of a nuclear protein (of as yet unidentified function) with hsp72 in human keratinocytes. Further characterization of hsp72-binding proteins will help to understand the function of hsp72 in human epidermis and its role in the protection of keratinocytes from environmental stress.

055

Corneodesmosin, a Component of Epidermal Corneocyte Desmosomes, Displays Homophilic Adhesive Properties

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Corneodesmosomes, the modified desmosomes of the uppermost layers of epidermis, play an important role in corneocyte cohesion. Corneodesmosin (Cdsn) is a secreted glycoprotein located in the corneodesmosomal core and covalently linked to the cornified envelope of corneocytes. Its glycine- and serine-rich NH_2-terminal domain may fold as structural motifs similar to the glycine loops (GL) described in epidermal cytokeratins and loricrin, and proposed to display adhesive properties. A chimeric protein comprising human Cdsn linked to the transmembrane and cytoplasmic domains of mouse E-cadherin was expressed in mouse fibroblasts to test the ability of Cdsn to promote cell-cell adhesion. Classic aggregation assays indicated that Cdsn mediates Ca^{2+}-independent homophilic cell aggregation. To assess the involvement of the GL domain in adhesion, full-length Cdsn, Cdsn lacking this domain, or this domain alone were expressed as GST-fusion proteins and were tested for protein–protein interactions by overlay binding assays. The results confirmed the homophilic interactions of Cdsn. Moreover, they indicated that its NH_2-terminal GL domain is sufficient but not strictly necessary to promote binding, suggesting that it is not the only domain bearing adhesive properties. Altogether, these results provide the first experimental evidence of the adhesive properties of Cdsn and of the involvement of its GL domain in cell adhesion.

056

Corneodesmosin is a Preferred Substrate of Both Stratum Corneum Tryptic Enzyme (SCTE) and Stratum Corneum Chymotryptic Enzyme (SCCE)

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Corneodesmosin (Cdsn) is an adhesive protein located in the extracellular part of the desmosomes in the upper layers of the epidermis. Synthesised by granular keratinocytes as a 52–56 kDa protein, Cdsn is progressively proteolysed during corneocyte maturation. At the epidermis surface, its cleavage which is necessary for desquamation to occur, only leaves intact the central domain of the protein, probably devoid of adhesive properties. The responsible proteases are unknown but SCCE and SCTE, 2 enzymes present in the stratum corneum (SC) and suggested to be involved in corneodesmosome degradation, are good candidates. To test whether Cdsn is a substrate of the enzymes, extracts of human epidermis were treated with epidermal SCTE and with either recombinant or epidermal SCCE. Treatments were performed at pH 7.4 and pH 5.6, the latter being closer to the physiological pH of the SC. Proteins were then immunoblotted with antibodies directed to Cdsn but also to desmogleins and desmocollins, the desmosomal cadherins which are also present in the extracellular part of the corneodesmosomes. At pH 7.4, both SCTE and SCCE degraded Cdsn. SCTE generated two immunoreactive fragments of 48 and 35 kDa, whereas SCCE generated several fragments from 48 to 15 kDa. Both enzymes also degraded desmocollins. However, desmogleins as well as most of the proteins of the extracts including involucrin, were unaffected. Identical results were obtained with the epidermal or recombinant SCCE. At pH 5.6, only Cdsn was degraded by SCTE, but somewhat less efficiently than at pH 7.4. Proteolysis of Cdsn by SCCE was also observed. The results demonstrate that Cdsn is a preferred substrate of both SCCE and SCTE *in vitro*, and suggest that the two proteases are involved in the proteolytic maturation of the protein in the SC. They support previous studies showing the importance of both enzymes in desmosome degradation and in desquamation.

057

Functional Analyses of Connexin Mutations in Keratinocytes

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The findings of germline connexin mutations in skin disease have shown that intercellular communication via gap junctions is an important mechanism by which the normal epidermis develops and differentiates. Distinct mutations in four connexins, Cx26, Cx30.3, Cx30 and Cx31, have been found either to underlie hearing loss and/or three types of hyperproliferative epidermal disease: Vohwinkel's syndrome, Hidrotic Ectodermal Dysplasia and Erythrokeratoderma variabilis. These genetic studies show that different connexin molecules and mutations have distinct junctional channel properties with regard to the co-ordination of epidermal differentiation. Tissue biopsies from patients in conjunction with cell culture models (both keratinocytes and HeLa cells) transfected with GFP tagged connexin fusion proteins are being used to study details of mutant connexins with regard to junction assembly and function. For example, immunohistochemical analysis of a palm biopsy taken from an affected site of an EKV patient harbouring the Arg42Pro mutation in Cx31 revealed cytoplasmic location of the Cx31 protein. GFP constructs of wildtype Cx31 and Cx31Arg42Pro mutant DNA were transfected separately into primary keratinocytes. 48 h post-transfection, the wtCx31/GFP fusion protein was localised at the plasma membrane of keratinocytes with the characteristic punctate staining and distribution of gap junction plaques between adjoining cells. In contrast to wtCx31/GFP, the Arg42ProCx31/GFP fusion protein was localised in the cytoplasm as demonstrated for endogenous Cx31 in affected EKV palm skin. These data show that the mutation prevents the protein reaching the plasma membrane and forming intercellular channels. Other connexin mutations such as M34T, D66H and R75W in Cx26, M190L in Cx30.3 and G11R in Cx30 in addition to cSNPs have been analysed in this way and will be described. These data show the effect of mutation on the processing of connexin proteins and will provide useful molecular tools towards understanding the effect of specific connexin mutations on gap junctional intercellular communication.

058

An Inducible Mouse Model for Epidermolytic Hyperkeratosis: Defining the Role of Stem Cells in Mosaic Skin Disorders

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Mosaic skin disorders are characterized by the presence of at least two genetically distinct cell populations from the same differentiation lineage within the same tissue. The mechanisms that lead to clinical mosaicism are poorly understood. It remains unclear, how certain genetic defects lead to a mosaic disease, whereas others never become apparent in the same tissue. To study the molecular and cellular basis of mosaicism, we established a mouse model for the autosomal-dominant skin blistering disorder, epidermolytic hyperkeratosis (EHK) which is characterized by a generalized and a mosaic form. This genetic model allows activation of a somatic K10 mutation in epidermal stem cells in a spatially and temporally controlled manner using an inducible Cre recombinase. Topical application of the inducer (RU486) to a circumscribed area of the skin resulted in a phenotype characteristic of mosaic diseases, with patches of affected and unaffected skin. The EHK lesions persisted after 3–5 topical applications of RU486, to date for over 12 months. Our results indicate that lack of selective pressure against certain mutations in epidermal stem cells leads to mosaic phenotypes. This finding has important implications for the development of new strategies for somatic gene therapy of dominant genodermatoses.

059

The *Elovl3* Gene is Required for Normal Development of Hair Follicles and Skin Barrier Function

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Esterified or as free acyl chains, very long chain fatty acids (VLCFA) are indispensable for the formation and function of the permeability layer of the skin. *Elovl3* is a tissue specific expressed gene whose product belongs to a highly conserved family of microsomal enzymes involved in the formation of VLCFA and sphingolipids from yeast to man. Here, we report a novel mechanism, which involves the *Elovl3* gene product in the formation of hair follicles and the function of skin barrier. Expression analysis of the *Elovl3* gene revealed a distinct expression in the sebocytes in the sebaceous glands and in the epithelial cells of the hair follicles. By disruption of the *Elovl3* gene by homologous recombination in mouse, we show that ELOVL3 participates in the formation of esterified VLCFA in the hair follicles and the sebaceous glands, and that these lipid compounds are essential for the barrier function of the skin. The *Elovl3*-ablated mice displayed a sparse hair coat and dry, atopic skin. The pilosebaceous system was hyperplastic and the hair lipid content was disturbed with exceptionally high levels of eicosenoic acid (20 : 1). A functional consequence of this is that *Elovl3*-ablated mice exhibited a severe defect in water repulsion and increased *trans*-epidermal water loss.

060

Inhibition of Androgen Receptor Expression in Human Sebocytes *In Vitro* by Antisense Oligonucleotides

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The effects of androgens on the skin are influenced by the amount of nuclear androgen receptor (AR) molecules available in skin cells. Androgen activity on the skin can be inhibited by systemic administration of compounds that exhibit strong AR affinity and antagonize binding of androgen molecules to AR. Androgens are the best-known stimulators of sebaceous gland activity, enhancing lipogenesis, proliferation and terminal differentiation of human sebocytes *in vivo* and *in vitro*. In this study we applied a new technology to realize the same purpose; we tested thioat- and ribosyl-antisense oligonucleotides against the AR. Antisense molecules are highly specific because they bind to mRNA targets at multiple levels of interaction at a single receptor site. To transfer antisense oligonucleotides into human SZ95 sebocytes we developed a cationic liposome-mediated transfection system with DOTAP. The transfection efficiency was assessed using FITC-labeled $(ACTG)_5$ random oligonucleotides that were detected within the nucleus. The expression of AR on mRNA and protein levels was investigated by semiquantitative RT-PCR and Western blotting. The transient transfection of SZ95 sebocytes with thioat (0.4 and 1.0 μM) and 2'-methyl-ribosyl (0.4 and 1.0 μM) antisense oligonucleotides resulted in a diminished protein expression of the AR after 24 h. After longer recovery times the AR protein expression in transfected SZ95 sebocytes reached again the level of native AR expression. The most successful transient control of AR expression was detected with 1.0 μM 2′-methyl-ribosyl antisense after 14 h and revealed a 87% inhibition of AR expression compared with native SZ95 sebocytes. The biological effect of several modified antisense-oligonucleotides was evaluated by estimating the proliferation pattern of androgen-challenged sebocytes treated with antisense-oligonucleotides.

061

Protein A Immunoadsorption: A Novel and Effective Treatment of Pemphigus

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Pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are autoimmune blistering diseases associated with antibodies to desmoglein (Dsg) 1 and Dsg 3. The pathogenic relevance of antibodies to these desmogleins has been demonstrated by passive transfer into neonatal mice where the intradermal injection of IgG, purified from the serum of PF or PV patients, induces acantholysis. Preadsorption of PV/PF IgG with recombinant Dsg3/Dsg 1 inhibits blister formation in the mice. Based on these data, we treated 4 patients (PV $n = 3$, PF $n = 1$) by immunoadsorption using a regenerable staphylococcal protein A adsorber which selectively removes immunoglobulins from the circulation. During each PA-IA, 2–3 plasma volumes (6–8 L) were processed resulting in a decrease of antibodies to Dsg 1/Dsg 3 by more than 90%. Three of our patients had been refractory to various treatment modalities and in 1 patient, PA-IA was chosen as the first treatment. All patients had high levels of circulating autoantibodies as detected by indirect immunofluorescence on monkey oesophagus and ELISA using recombinant desmoglein 1 and 3. Within a period of 4–24 weeks, skin lesions cleared completely in all patients. Concurrently, levels of circulating antibodies became negative in 2 patients and declined markedly in the other 2 cases. Three patients have now been free of skin lesions during a follow-up period of 8–84 weeks. At the same time, intervals between PA-IA were extended from initially 1 week to 3 weeks; in 1 case, PA-IA has now been tapered off for more than 1 years without relapse. In all patients, the accompanying dose of oral methylprednisolone (initially 0.5 mg per kg body weight per d) could be greatly reduced. In summary, our data suggest that PA-IA is a rational, effective, and safe therapy for pemphigus and warrant the wider use of PA-IA for this indication.

062

Functional Thyroid Peroxidase Autoantibodies of IgE Class in Patients with Chronic Urticaria

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Chronic urticaria (CU), a frequent dermatosis caused by autoimmunity, allergy, chronic infection or pseudoallergy among other mechanisms, has been reported to be preceded by autoantibody-mediated autoimmune diseases including thyroid autoimmunity. Speculating that thyroid autoantibodies may be involved in the pathogenesis of CU, we have characterized the prevalence, Ig class pattern and function of antibodies against thyroid peroxidase (TPO) in patients with CU. Out of 116 CU-patients tested, 23 (19.8%) exhibited increased IgG-antibodies to TPO (IgG-TPO-Ab), as assessed by ELISA ($p < 0.005$ compared to 5.6% in healthy controls). Interestingly, virtually all IgG-TPO-Ab-positive (88%, $n = 17$), but none of 33 IgG-anti-TPO-negative CU-patients, also exhibited TPO-Ab class IgE ($p < 0.005$), as assessed by immunoprecipitation. To test whether these IgE-TPO-Ab are functional, mouse mast cells (MC) expressing human FceRI were loaded with serum IgE-fractions of IgE-TPO-Ab-positive or IgE-TPO-Ab-negative CU-patients. MC degranulation was determined after stimulation with human recombinant TPO (rhTPO) by measuring release of β-hexosaminidase. IgE-TPO-Ab-pretreated MC showed significantly increased release of β-hexosaminidase ($17.3 \pm 1.2\%$) after stimulation with rhTPO as compared to MC pretreated with IgE-fractions of IgE-TPO-Ab-negative CU-patients ($3.4 \pm 0.2\%$, $p < 0.05$) or IgE-TPO-Ab-pretreated MC stimulated with vehicle ($4.7 \pm 0.3\%$, $p < 0.05$). Our findings confirm a high incidence of increased thyroid antibodies in patients with CU and show for the first time that a significant percentage of CU-patients exhibits "autoallergic" TPO-Ab of IgE class, capable of activating MC in the presence of TPO. Thus, "allergy to self" may be a novel mechanism involved in the pathogenesis of chronic urticaria.

063

Low-Dose Long-Term IL-10 Treatment Prolongs the Disease-Free Interval in Psoriasis

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The ability of intermediate-term IL-10 therapy to reduce the severity of exacerbated psoriasis has been demonstrated recently. The objective of this study was to determine the safety and potency of long-term, low-dose IL-10 application in order to prolong the duration of psoriasis remission. We performed a placebo-controlled, double-blind, phase II clinical trial using rhIL-10 in patients with chronic plaque psoriasis in remission. Patients received nonlesional subcutaneous injections with either IL-10 (10 μg per kg body weight; $n = 8$) or placebo ($n = 11$) 3 times per week for 4 month. Immunological investigations (plasma parameters, mitogen-induced lymphokine secretion in whole blood) were performed monthly. Overall the treatment was well tolerated. Only moderate and nonpersisting effects on hematopoetic cells were observed. Renal and hepatotoxicity were excluded. One patient each from the placebo and IL-10 treatment groups prematurely drop out of the study for private reasons and were excluded from the further analyses. In the placebo group almost all patients (9/10–90%) showed a relapse of psoriatic disease during the 4 month observation period. In contrast to this, only 2 out of 7 patients (26.6%) relapsed in the IL-10-treated group. The mean relapse-free interval during study time was 102 days in the IL-10 group in comparison to 66 days in the placebo group. In Kaplan Meier analysis the relapse incidence was significantly different in the IL-10 and the placebo groups ($p = 0.022$ Log Rank test). In accordancy with results from therapeutic psoriasis studies, IL-10 treatment led to in tendency lower IFN-γ and higher IL-4 secretion capacities. Moreover, for all patients a significant negative correlation between the *ex vivo* mitogen-induced IL-4 secretion and PASI was demonstrated ($r = 0.36$, $p < 0.01$). Remarkably, the 2 psoriasis patients who relapsed in the IL-10 group lacked immunological signs for IL-10 reactivity. Our data suggest that low-dose, long-term therapy with rhIL-10 is safe and clinically effective to prolong the disease-free interval in psoriasis. Consequently, its value should be further determined in larger trials including dose-finding studies. Moreover, the value of long-term IL-10 treatment for the prevention of re-exacerbation of other inflammatory disorders with a similar immunological profile, as inflammatory bowel disease, rheumatoid arthritis and transplant rejection, should be analyzed.

064

Neutralization of the Dendritic Cell Specific Costimulatory Molecule, Dectin-2, Breaks UV-Induced Tolerance

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Ultraviolet (UV) light abrogates contact hypersensitivity (CHS) responses and induces hapten-specific tolerance. Although this phenomenon has long been appreciated, molecules specifically involved in this pathway still remains to be shown. We could show currently that dectin-2 (Dec2), the dendritic cell-specific C-type lectin receptor, selectively mediates UV-induced tolerance, but not the induction of CHS, when Dec2 is functionally neutralized before sensitization. Since the establishment of tolerance needs specific signals at resensitization with haptens, i.e. the Fas/FasL system, we questioned whether Dec2 confers signals to establish tolerance in a specific way. Therefore, C3H/HeN mice that were exposed to low doses of UV light over 4 days (d) and hapten-sensitized through radiated-skin area with dinitrofluorobenzene showed profound inhibition of the CHS response. UV-treated mice resensitized 14 d after the first challenge displayed hapten-specific tolerance. Intravenous (*i.v.*) injection of soluble Dec2 (sol-Dec2), an antagonist of cognate Dec2 expressed on dendritic cells, before resensitization abrogate UV-induced tolerance, however, tolerance is not affected at all when sol-Dec2 is *i.v.* injected before rechallenge, demonstrating that neutralization of Dec2 breaks UV-induced tolerance. As control experiments, mice that were not UV-exposed but hapten-sensitized were resensitized and rechallenged showed the vigorous ear swelling response, which was not affected upon injection of sol-Dec2 before resensitization or rechallenge. Adoptive transfer of T cells from regional lymph nodes of UV-exposed mice treated with sol-Dec2 had no effect on the CHS response in recipient mice, whereas the transfer of T cells from UV-treated mice inhibited the immune response. These findings indicate that sol-Dec2 can selectively breaks UV-induced tolerance without affecting effector routes of the CHS response, thereby demonstrating that Dec2 plays a central role in generation of immunological tolerance by UV light.

065

A Crucial Role of Dectin-2 to Establish UV-Induced Tolerance by Supressor T Cells

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Ultraviolet (UV) light abrogates the contact hypersensitivity (CHS) response and induces hapten-specific tolerance. In this context, it is well accepted that suppressor T cells play a pivotal role in the induction of tolerance. Biological significance of suppressor T cells is best demonstrated that those cells may reproduce the UV-induced immunosuppressive phenotype upon transfer to naïve animals by activating molecules, i.e. Fas/Fas ligand system, in T cell-transferred recipient animals. However, it still remains to be shown how signals in recipients are activated. Therefore, we focused on Dectin-2 (Dec2), the dendritic cell specific C-type lectin receptor with costimulatory functions. C3H/HeN mice that were first exposed to low doses of UV for 4 consecutive days (d) and sensitized with dinitrofluorobenzene through UV-radiated skin areas exhibited profound suppression of CHS as compared to those only sensitized and challenged, but not UV-exposed, indicating that the induction of UV-induced immunosuppression. T cells were isolated from those animals and transferred to naïve recipients, resulting in marked suppression of CHS in recipient mice. To address whether Dec2 is involved in this process, naïve mice that were intravenously (*i.v.*) injected with sol-Dec2, an antagonist for cognate Dec2 expressed on dendritic cells or with its irrelevant control protein, DFHR, were transferred with T cells from UV-tolerized or non-UV exposed animals 3 h after the injection. 1 d after the transfer those mice were sensitized and challenged. While immunosuppression of CHS induced in transferred-animals was not affected at all upon *i.v.* injection of DFHR, which, however, was significantly restored by *i.v.*injection with sol-Dec2. In addition, transfer of T cells from non-UV-exposed, but hapten-sensitized animals produced vigorous the ear swelling response in recipients, which was not affected upon *i.v.*injection of DFHR nor of sol-Dec2. Together, our present study demonstrates that Dec-2 selectively plays a decisive role in the induction of tolerance by suppressor T cells. Furthermore, this is the first demonstration of the presence of Dec2-dependent signaling pathways from suppressor T cells to dendritic cells in order to establish immune tolerance by UV light.

066

Down-Regulation of HLA Class I Transcription in Advanced Melanoma Metastasis

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The HLA class I complex is operative in almost all cells and allows the immune system to monitor all tissue for the presence of viral infections or tumors. The presentation of endogeneous synthesized peptides in association with HLA class I allows the activation of $CD8^+$ lymphocytes. Tumor cells often fail to present antigenic peptides resulting in an immune escape of metastasizing cells. We try to illuminate possible molecular mechanisms leading to a lack of antigen presentation. A panel of 32 malignant melanoma short-time cultures were analyzed geno- and phenotypically for HLA gene expression. Transcriptional activities of HLA-A, -B and -G genes were monitored using new quantitative LightCycler™ PCR technology and compared. Since in 30 metastasis the genomic analysis revealed the presence of the corresponding HLA class I allospecificities despite reduced protein expression on the cell surface we explored the possibility of a transcriptional gene regulation. There was no correlation observed between down-regulation of HLA-A and HLA-B but between gene expression and gene transcription levels. Additionally, an overall reduction of HLA class I gene transcription in melanoma metastasis was observed over the time for individual patients. Therefore, we postulate a transcriptional regulation of the HLA class I gene expression in melanoma involved in immune escape processes. The documented HLA down-regulation in malignant melanoma metastasis suggests a central role for HLA in tumor progression and limits the efficacy of specific immunotherapy.

067

Loss of E-Cadherin Expression in Melanoma Cells Involves Upregulation of the Transcriptional Repressor Snail

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Malignant transformation of melanocytes frequently coincides with loss of E-Cadherin expression. We could show that loss of E-Cadherin in melanoma cell lines does not involve mutations in the E-Cadherin gene, promoter methylation or alterations in expression of AP-2 transcription factors as suggested previously. In a panel of different melanoma cell lines E-Cadherin expression was negatively regulated by up-regulation of the transcription factor Snail. In comparison to primary human melanocytes, where Snail expression was not detected by RT-PCR, significant expression was found in all eight melanoma cell lines. In parallel, Western blot and RT-PCR analysis revealed strong reduction of E-Cadherin expression in the melanoma cells. Consistently, transient transfection of a Snail expression plasmid into human primary melanocytes led to significant down-regulation of E-Cadherin, whereas transient and stable transfection of an antisense snail construct induced reexpression of E-Cadherin in Mel Ju and Mel Im melanomas. In summary, we conclude that activation of Snail expression plays an important role in down-regulation of E-Cadherin and tumorigenesis of malignant melanomas.

068

Increased Susceptibility to Skin Carcinogenesis in Mast Cell-Deficient Mice

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Mast cells (MC) have been reported to accumulate at sites of cutaneous tumor formation where they are suspected to be involved in growth promotion and angiogenesis. To test whether MC modulate the development of epidermal tumors, MC-deficient Kit^{W}/Kit^{W-v}-mice were subjected to a two-stage skin carcinogenesis model. Kit^{W}/Kit^{W-v}-mice and normal *Kit*+/+-mice were treated topically with a carcinogen (DMBA, single application) followed by a promotor (TPA, 2x/week for 17 weeks). Papillomas were first found in both genotypes 9 weeks after the start of promotion. Unexpectedly, Kit^{W}/Kit^{W-v}-mice proceeded to develop tumors significantly faster than normal *Kit*+/+-mice: After 12 weeks of treatment 82% of Kit^{W}/Kit^{W-v}-mice ($n = 28$), but only 35% of *Kit*+/+-mice ($n = 40$), showed ≥ 1 papilloma (data pooled from 3 independent experiments, $p < 0005$). Interestingly, after 12 weeks of treatment Kit^{W}/Kit^{W-v}-mice also exhibited more papillomas per treatment site (3.1 ± 0.4 papillomas per mouse) as compared to *Kit*+/+-mice (1.4 ± 0.4 papillomas per mouse, $p < 0.02$). Suspecting that MC release products required for resistance to *de novo* carcinogenesis, we tested whether MC undergo degranulation during repeated TPA treatment. As assessed by histomorphometric analysis, virtually all subepidermal MC treated for 16 weeks exhibited signs of extensive ($70 \pm 7.5\%$) or moderate ($29 \pm 5\%$) degranulation, while subcutaneous MC were activated to a markedly lesser extent ($40 \pm 10.5\%$ not degranulated, $p < 0.05$). These findings suggest that activated MC confer resistance to skin tumor development. Our data also extend the view of MC as salient skin sentinels in the context of innate immune responses.

069

Impaired Control of Skin Infection with Pseudomonas Aeruginosa in the Absence of Mast Cells

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We and others have recently shown that peritoneal mast cells (MC) play a central role in the initiation of efficient innate immune responses in acute septic peritonitis. To test whether skin MC populations contribute to resistance and containment of bacterial infections, we subjected genetically MC-deficient Kit^{W}/Kit^{W-v}-mice to skin infections with *Pseudomonas aeruginosa* (PA). Female Kit^{W}/Kit^{W-v}-mice and normal+/+ littermates (*Kit*+/+) were injected subcutaneously with $\sim 8 \times 10^6$ colony forming units of PA (0.1 mL, shaved lower back), and the size of skin lesions, characterized by infiltration and subsequent necrosis, was assessed by planimetric analysis (every 2–6 h, for 72 h). Developing skin lesions in Kit^{W}/Kit^{W-v}-mice were markedly larger (up to 3 fold) than in *Kit*+/+ mice at all time points. *Kit*+/+ skin lesion size reached a maximum at 14 h after infection ($0.7 \pm 0.2\,cm^2$ vs. $1.4 \pm 0.7\,cm^2$ in Kit^{W}/Kit^{W-v}), while lesions in Kit^{W}/Kit^{W-v}-mice continued to increase for 36 h following infection ($1.6 \pm 0.6\,cm^2$ vs. $0.6 \pm 0.1\,cm^2$ in *Kit*+/+ at 36 h). Virtually all Kit^{W}/Kit^{W-v}-mice, but no *Kit*+/+ mouse, exhibited piloerection, a sign of systemic infection, as soon as 12 h and up to 50 h after PA-injection, indicating systemic dissemination of PA. Therefore, clinical symptoms of sepsis were assessed with the help of a clinical disease score, grading the degree of spontaneous activity, social behavior and flight reaction (maximum value of 3). Clinical disease after injection of PA in Kit^{W}/Kit^{W-v}-mice started earlier and was more severe than in *Kit*+/+ mice (Kit^{W}/Kit^{W-v}: 1.8 ± 0.2 vs. *Kit*+/+: 0.7 ± 0.3 at 2 h, $p < 0.05$). Clinical disease score values in *Kit*+/+ mice, but not in Kit^{W}/Kit^{W-v} mice, started to drop 12 h after infection (0.5 ± 0.2 vs. 1.7 ± 0.3 in Kit^{W}/Kit^{W-v} at 14 h, $p = 0.02$), indicating that recovery from PA-induced morbidity is impaired in MC-deficient mice. Our data suggest that MC (1) control the size of skin lesions in PA infection and (2) promote containment of PA at the site of skin infection. These findings implicate MC as potential effector cells in the context of bacterial skin infections.

070

Association Between p53 Codon 72 Polymorphism and Susceptibility to Skin Cancer

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Up-regulation of p53 protein induces either growth arrest or apoptosis and this is signalled from a highly conserved p53 domain between codons 64–92, where a functional polymorphism results in either proline (p53–72P) or arginine (p53–72R) at position 72. Preliminary studies suggest that p53–72R may be a risk factor for cervical cancer and preferential mutation and retention of the p53–72R allele has been demonstrated in other squamous cell cancers. We have examined the relationship of the polymorphism and nonmelanoma skin cancer by determining the association with susceptibility to sunburn (as assessed by skin phototype and minimal erythemal dose), p53 mutations, loss of heterozygosity and human papillomavirus status. We found a significant relationship between p53–72R and susceptibility to sunburn ($p = 0.0001$ for trend) and between p53–72R and nonmelanoma skin cancer in renal transplant recipients ($p = 0.01$) but not in immunocompetent patients compared to skin type matched controls. p53 sequence data revealed mutations in 30/70 (42.9%) nonmelanoma skin cancers, 28 (93%) of which were in the p53–72R allele. Loss of heterozygosity occurred more frequently in p53–72RP than in p53–72RR tumours ($p = 0.0001$) with preferential loss of p53–72P in heterozygotes ($p = 0.016$), irrespective of the mutant status of the concomitant allele. Together these data infer functional differences between polymorphic forms of p53 that are likely to be relevant to skin carcinogenesis.

071

Mutations of the INK4A-arf and p53 Tumor Suppressors Genes in Genital Carcinoma

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The INK4A-ARF locus (encoding the P16INK4a and P14ARF proteins) and the p53 genes are important cell cycle regulators. They are inactivated by UV-induced mutations in skin carcinomas from both sporadic and XP patients. In genital cancer p53 can be inactivated by either mutation or interaction with oncogenic HPV E6 protein. Very few studies have looked at the status of p16INK4A-ARF in genital tumors. In this work we have looked for mutations in the p16INK4A-ARF locus (exon 1α, 1β, 2) and the p53 gene (exon 4–8) by PCR SSCP and sequencing in genital cancers ($N = 10$), 7 developped on lichen sclerosus (LS) and 3 on HPV-related genital lesions. We also analysed precursor lesions LS ($N = 22$) or condyloma ($N = 3$), and normal surrounding genital skin ($N = 14$). Our preliminary results show that both p53 and p16INK4A-ARF are frequently mutated in genital cancer as mutations are present in at least 60% and 40% of the tumors, respectively. Three additionnal abnormal band shifts are currently analyzed. Additionnally, cumulation of mutations in both INK4A-ARF and p53 are so far observed in 40% of the tumors strongly suggesting a selection advantage of double mutants. Characterization of the mutations showed 3 C > T transitions and 4 tranversions for the p53 gene (in exons 5, 7 and 8), a mutational pattern radically different from the one of UV induced skin tumors. For the INK4A-ARF locus only C > T transitions were observed in exon 2 common between p16INK4A and p14ARF genes, with a hot spot on codon 80 (Arg80Ter). Conversly no mutation was observed in precursors lesions and normal skin. Immunohistochemical localization of the p53 protein was performed in 3 tumors and in 22 LS and showed stabilisation of the p53 protein in 66% and 27%, respectively. These results show that both p16INK4A-ARF locus and the p53 gene are important molecular targets in genital carcinogenesis. They appear as late even in tumor progession as they are found mutated mostly in cancer and not in prescursor lesion. Association for inactivation of both the p16INK4A-ARF locus and the p53 genes seem to be an important event in genital skin carcinogenesis.

072

The Antimalarial Artesunate Acts Also as a Potent Anticancer Drug via both p53-Dependent and -Independent Pathways

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Artesunate, a semisynthetic derivative of artemisinin, the active compound of the plant *Artemisia annua*, shows remarkable activity against otherwise multidrug resistant *Plasmodium falciparum* and *vivax* malaria. Artesunate has now been analyzed for its anticancer activity against 55 cell lines of the National Cancer Institute Human Tumor Drug Screen Panel. Artesunate, in comparable molar ranges as established cytostatic drugs, was most active against leukemia and colon cancer cell lines. Intermediate GI50 values were obtained for most other cell lines, including otherwise chemoresistant tumors like renal cancer and melanoma. Interestingly, MDR1/P-glycoprotein overexpressing multidrug-resistant cancer cells did not show cross-resistance to artesunate compared to sensitive parental cells, and no correlation with MDR1 mRNA expression or MDR1 transporter functionality was observed. Moreover, GI50 values were identical among cell lines with wild-type and mutated p53 tumor suppressor protein as well as induced or uninduced $p21^{CIP1}$ CDK inhibitor. This unusual quality of artesunate was further analyzed in a $p53^{+/+}p21^{+/+}$ cancer cell line and its respective knockout counterparts. Artesunate, in a time- and dose-dependent manner, potently induced cell cycle arrest, as judged by profound inhibition of the retinoblastoma G_1/S pathway, and apoptosis in $p53^{+/+}$ as well as in isogenic $p53^{-/-}$, $p21^{-/-}$, and 14–3-3 $\sigma^{-/-}$ cells. This points to overlapping but distinct pathways at the G_1/S checkpoint activated by artesunate. Taken together, our results and the low toxicity documented in large clinical malaria studies are clues that artesunate may be a promising candidate for the therapy of tumors resistant to conventional chemotherapy, including melanoma.

073

Determination of Sequence Diversity in the Gene for Collagen XVII in Autoimmune Blistering Disease Patients Shows Conservation of the NC16A1 Domain

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The autoimmune blistering diseases of the skin are characterised by autoantibody attack of type XVII Collagen (BP180). Collagen XVII forms an adhesion complex, which extends from the basal keratinocytes into the basement membrane zone. Autoantibody attack is directed at the NCA16 domain positioned between the membrane-spanning domain and the long extracellular collagenous tail of the molecule. Sequence variation of the gene which codes for collagen XVII (Collagen 17A1) was determined in a panel of 29 Caucasian individuals using DHPLC analysis on the WAVE ™ DNA Fragment Analysis System (Transgenomic) and DNA sequencing of the exons which were shown to have changes. Of the 29 individuals 11 had bullous pemphigoid, 8 had cicatricial pemphigoid and 10 had linear IgA disease. All had autoantibodies to the basement membrane zone specifically collagen XVII. 19 polymorphisms were identified in 29 individuals. All were single base substitutions. The majority of variable sites were found in noncoding regions. Of the variable sites characterised in coding regions; four had variations that resulted in amino-acid substitutions and four were silent substitutions. No polymorphisms were found in exon 18, which codes for the NCA16 domain. All previously reported polymorphisms were confirmed. These results show that although the gene for collagen XVII is polymorphic, the region encoding for NCA16 does not tolerate polymorphisms, which may alter the structure of the encoded molecule. This suggests the structure of this region is strictly conserved to maintain its function.

074

Sera from Patients with Cicatricial Pemphigoid React with both C- and N-Terminal Sites on the BP180 NC16A Domain, in Contrast to Sera from most Bullous Pemphigoid and Pemphigoid Gestationis Patients

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Bullous pemphigoid (BP), pemphigoid (herpes) gestationis (PG), cicatricial pemphigoid (CP), and lichen planus pemphigoides (LPP), are autoimmune subepidermal bullous diseases that are characterized by circulating autoantibodies to the transmembrane hemidesmosomal protein BP180/type XVII collagen. Previous studies demonstrated that the majority of patients with BP, PG, and LPP show antibodies to an immunodominant, membrane-proximal noncollagenous domain (NC16A) on the extracellular portion of BP180. By the use of nonoverlapping peptides of the NC16A domain, we previously demonstrated that autoantibodies from BP and PG patients mainly react with epitopes clustered within the N-terminus of this immunodominant site of BP180; antibodies from patients with LPP also recognized the C-terminal portion of NC16A. However, some of these results had been obtained indirectly by preadsorption studies. The aim of the present study was to analyze the fine specificity of autoantibodies to NC16A in sera from patients with CP and to compare their reactivity with antibodies from BP, PG, and LPP patients using a series of new overlapping fragments covering the entire NC16A domain. We confirm that BP and PG sera mainly react with N-terminal epitopes of NC16A, whereas sera from patients with LPP also bind to C-terminal portions of this domain. Interestingly, out of 10 patients with CP, seven reacted with NC16A; within NC16A, these sera bound to both C-terminal fragments and a N-terminal epitope right next to the cell membrane. Our data demonstrate a heterogeneous binding pattern of autoantibodies to BP180 NC16A in patients with CP.

075

False-Negative Results in Immunoblot Assay of Serum IgA Antibodies Reactive with the 180-kDa Bullous Pemphigoid Antigen (BP180): the Importance of Primary Incubation Temperature

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One of the methods to assess the antigen specificity of circulating antibodies in acquired immunobullous dermatoses is immunoblotting. In fact many of the target antigens known today have initially been discovered by this technique. This success has made immunoblotting into a widely used diagnostic tool for discriminating between diseases that show similar patterns by serum immunofluorescence, but in which the actual target antigens may differ. Since immunoblotting seems a relatively simple technique with easy results, many laboratories use it for identification of the antigen-specificity of serum autoantibodies but protocols and assay conditions may differ significantly between laboratories. For instance the incubation temperature during the first step, the contact between filter-bound antigen and patient serum, is in the literature often given as ''ambient'' or ''room'' temperature. In the current study we show that in IgA-immunoblotting assays for BP180 it particularly is this first step which may be crucial for obtaining a reliable result. Being faced with irreproducible results in seemingly identical assays we carefully evaluated each step of the protocol. Here we show that increasing the primary incubation temperature from room temperature (19–20°C) to 37°C results in a dramatic improvement of IgA-binding to BP180. A test panel of 11 sera was selected from patients who, in skin or conjunctival biopsies, had linear EBMZ-IgA deposits, which sometimes were accompanied by linear IgG. Based on clinical presentation and immunopathological criteria these patients suffered from bullous pemphigoid ($n = 1$), cicatricial pemphigoid with or without ocular involvement ($n = 5$), ocular cicatricial pemphigoid ($n = 4$) or linear IgA bullous dermatosis ($n = 1$). This test panel was assayed by immunoblot at different primary incubation temperatures. Where at room temperature no or incidental binding to BP180 was observed, IgA from 10 out the 11 sera clearly bound BP180 when incubated at 37°C.

076

***Saccharomyces cerevisiae* and *Pichia pastoris* as a Novel Source for Production of Human Bullous Pemphigoid Antigen 230 (BP230)**

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BP230 is a protein of 2649 amino acids of the plakin family, that was originally identified as a major autoantigen in bullous pemphigoid (BP). The protein is a constituent of junctional complexes called hemidesmosomes and is involved in cytoskeletal organization. To better define its role as cytoskeletal linker and as target antigen of BP, we have attempted to produce BP230 and its entire 770 residue tail in fusion with the FLAG™ peptide (F) (F-BP230$^{1–2649}$) and GST (GST-BP230$^{1880–2649}$), respectively, using yeasts *S. cerevisiae* and *P. pastoris* expression systems. Immunoblot analysis of extracts of yeasts transformed with expression vectors for BP230 and the deletion mutant demonstrated that *P. pastoris* was not able to produce F-BP230 $^{1–2649}$, whereas *S. cerevisiae* expressed minor amounts of the protein, which appeared proteolytically degraded. In contrast, both *S. cerevisiae* and *P. pastoris* produced GST-BP230$^{1880–2649}$. A time course analysis in *S. cerevisiae* revealed that the optimal time of induction was 48 h. Furthermore, the level of production of GST-BP230$^{1880–2649}$ in *S. cerevisiae* was higher than in *P. pastoris* (42.5 ng vs. 5 ng per 100 μg of extracts) and higher than the amount of native BP230 in cultured keratinocytes. The expressed GST-BP230$^{1880–2649}$ was only partially soluble in buffers containing 6 M urea and/or 1% Triton-100, making its purification difficult. Finally, when the reactivity of BP sera against yeast extracts was assayed, 12 of 17 (71%) BP sera were found to be reactive with GST-BP230$^{1880–2649}$, while no reactivity was observed with 6 of 6 (0%) sera from normal volunteers. In conclusion, S. cerevisiae yeast represents a novel source for the production of truncated forms of BP230, but not for the wild-type protein. The expressed recombinants may be useful tools for the diagnosis of BP and related disorders.

077

Comparison of Alterations of Basement Membrane Zone in a Course of Pemphigoid and Epidermolysis Bullosa Acquisita: Three Dimensional Reconstruction of BMZ in Laser Scanning Confocal Microscopy

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Pemphigoid and epidermolysis bullosa acquisita (EBA) are characterized by the presence of *in vivo* bound and circulating anti basement membrane zone (BMZ) antibodies directed against different antigens localized in upper part of lamina lucida and in sublamina densa regions, respectively. The alterations of BMZ during the process of the diseases leading eventually to blister formation. The present study was undertaken to compare alterations of various regions of the BMZ in pemphigoid and EBA using laser scanning confocal microscopy (LSCM). Biopsies taken from perilesional skin of pemphigoid and EBA were cut into 20 mm thick slides followed by immunofluorescence labeling with antibody against β-4 intergin (lamina lucida marker-upper part), antibody against epiligrin (lamina lucida marker-lower part), antibodies directed against collagen type IV and N-terminal end of collagen VII (lamina densa-LD markers), antibody directed against C-terminal end of collagen type VII (sublamina densa-SLD marker). Three dimensional reconstruction of various regions of BMZ was performed by computer programme integrated with LSCM. Our studies revealed numerous invaginations of BMZ on the level of lamina lucida and lamina densa in a case of pemphigoid. However continuous character of BMZ was preserved. In EBA case, in contrast to pemphigoid, discontinous staining on the level of SLD region was noticed and large clumps of collagen VII vertically orientated to the BMZ extended into the deep dermis were observed. Our study have shown that alteration of BMZ in pemphigoid mainly refers to lamina lucida whereas in EBA to sublamina densa region. The alterations of different regions of BMZ in pemphigoid and EBA could be responsible for discrepancy in the level of blister formation and course of the diseases.

078

Mixed Antibody Response in Paraneoplastic Pemphigus

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The increased awareness of paraneoplastic pemphigus (PNP) as a clinical entity has resulted in larger series of patients becoming available for study. Our group comprised 7 patients with PNP, 3 associated with chronic lymphocytic leukaemia, 1 with follicular lymphoma, 1 with Castleman's disease, 1 with non-Hodgkin's lymphoma, and 1 patient with metastatic laryngeal carcinoma. Immunohistochemistry was used to ascertain the autoantibody and complement profile (IgG/IgA/C3) in these 7 patients, and immunoblotting on keratinocyte and/or epidermal skin extracts to determine the specific antigens targeted. All 7 patients fulfilled the clinical, histological and immunopathological criteria for PNP with I/C staining by DIF, cell surface and BMZ staining by IMF, all showed positive IMF staining on rodent bladder, and bound 2 or more of the plakin family of PNP antigens. In addition to IgG and C3, PNP was also strongly associated with IgA antibodies. Four patient's sera showed IgA cell surface and BMZ binding by IMF, 5 sera bound the 190 kDa antigen and 1 the 170 kDa antigen by immunoblotting. In addition 2 sera reacted against the 250 kDa antigen. The single patient with a negative IgA response by IMF and blot analysis was undergoing immunoglobulin replacement fortnightly for variable immunodeficiency. The presence of IgA did not appear to be related to the clinical picture or treatment. Our results suggest that the IgA plays a significant role in the immune response in many PNP patients, and emphasises the heterogeneity and complexity of the disease.

079

CD8 Lymphocytes are Involved in the Pathogenesis of Acantholysis *In Vivo*

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Pemphigus Vulgaris (PV) is an autoimmune bullous skin disease characterized by antibodies directed against the desmosomal cadherin desmoglein-3. Recent studies have demonstrated a role for T lymphocytes in the pathogenesis of acantholysis. Activated T cells are present within PV lesions, and T-cell derived cytokines have been shown to be involved in acantholysis. A selective epidermal accumulation of activated CD8+ T cells has been found in paraneoplastic pemphigus where apoptosis has been identified as a key mechanism of keratinocyte death. It has been also suggested that cell-mediated cytotoxic reactions are probably related to enhanced proteolytic activity in the site of bullous eruption in PV. To further delineate the potential role of CD8 lymphocytes in the pathogenesis of acantholysis, we performed passive transfer experiments with PV IgG in gene targeted mutant mice. Our results demonstrated that CD8 deficient mice are significantly less sensitive to the development of PV lesions than wild type mice. These data indicate a role for CD8 lymphocytes in PV acantholysis and suggest that cytotoxic reactions leading to apoptosis of keratinocytes may play a role in the development of lesions in PV.

080

CD40-CD40L Expression in Lesional and Unaffected Skin of Pemphigus

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CD40 is a surface antigen of B and T cells, mononuclear and antigen-presenting cells (APCs), endothelial cells and some epithelial cells that interacts with a specific ligand (the so called CD40L) which is only transiently and slightly expressed by normal T cells, but conversely preferentially expressed by CD4+ activated T lymphocytes. The CD40-CD40L signaling process is a pivotal component of multiple immunoregulatory pathways, such as the up-regulation of IgE synthesis or the *in vivo* priming of Th1 cells via the stimulation of IL12 secretion by APCs. In this study we tried to investigate if CD40-CD40L ligation, that usually represents a step of antigen presentation in limph nodes, could have a role in blister formation of pemphigus. In particular, we supposed it could promote the ''cognate interaction'' between T and B cells, leading to a skin model in which humoral and cellular immunity co-operate to the pathomechanism of the disease. To this purpose, an immunohistochemical technique (APAAP) using monoclonal antibodies (MoAbs) for CD40 and CD40L was performed on cutaneous biopsies taken from lesional and healthy skin of 6 pemphigus patients (3 affected by pemphigus vulgaris -PV- and 3 by pemphigus foliaceus -PF-). Furthermore, healthy subjects were used as negative control. In lesional skin of pemphigus patients, a focal perivascular staining for CD40L was found in two biopsies (1 PV, 1 PF), while two patients (2 PF) showed only groups of 3–4 big CD40L positive(+) cells localised in the upper dermis. The epidermal staining was always negative. CD40+ cells were detected in the basal layer of the epidermis and a positive staining was also found in the vascular and perivascular dermal sites of all patients. In unaffected skin, a weak positive staining for CD40L was demonstrated in the perivascular dermal areas. An intense staining for CD40 was evident on the epidermal cells. In all biopsies CD40+ cells were also found around dermal vessels and appendages, and in one case, we demonstrated CD40+ cells even scattered in the upper dermis. On the contrary, we did not find any CD40+ or CD40L+ cell in the biopsies from healthy donors. Taken together, our results point out a higher expression of both CD40-CD40L in lesional than in healthy skin. It might be conceivable therefore the activation of these antigens plays an additional pathogenic role to the production of autoantibodies and following immuno-mediated flogosis of pemphigus.

081

Atypical Bullous Skin Disease Associated with Sjögren's Syndrome

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In unusual circumstances, patients with systemic autoimmune diseases can develop subepidermal blisters, and one of the mechanisms whereby this occurs is through the development of autoantibodies against the type VII collagen. A 56-year-old Caucasian woman with primary Sjögren's syndrome developed a subepidermal bullous eruption, which responded well to medium-dose oral prednisolone therapy. Histology on the perilesional skin revealed subepidermal cleavage with edema formation in the papillary dermis, and polymorphic infiltration perivascularly. Direct immunofluorescence examination demonstrated linear IgG and C3 deposition along the basement membrane zone. On indirect immunofluorescence, the circulating IgG autoantibodies from the patient's serum labeled exclusively the dermal side of salt-split normal human skin. Immunoblotting examination on dermal protein extracts revealed circulating autoantibodies against the intact tissue type VII collagen in the patient's serum. Circulating autoantibodies against specific bullous pemphigoid antigens (BP230 and BP180) were not detected. We suggest that the presence of antitype VII collagen autoantibodies was directly connected with the blistering skin eruptions in the patient (possibly a generalized inflammatory EBA) and these autoantibodies may contribute to the initiation of bullous skin symptoms in other autoimmune disorders.

082

Tissue Transglutaminase (TGc) is a Minor Antigen in Autoimmune Disease Independent of Gluten Sensitive Enteropathy

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TGc is the major autoantigen of coeliac disease and dermatitis herpetiformis. To characterize its wider autoantigenic role, sera from patients with other disorders were analyzed. Sera from 304 patients with autoimmune diseases and 122 patients with diseases involving organ specific enhanced cell death were compared to those from 39 patients with coeliac disease and 56 with dermatitis herpetiformis and 84 samples from people with diseases of nonautoimmune origin or without known disease. IgA antibodies against tissue transglutaminase were detected using human and guinea pig antigens in an ELISA system. Anti-gliadin and antiendomysium antibodies were also determined. Fourty nine percent of sera from patients with autoimmune disorders reacted with the human TGc. However, no association of these conditions with gluten sensitive enteropathy could be shown. Healthy controls were negative. While IgA antibodies against TGc are a major feature in coeliac disease and dermatitis herpetiformis, they also occur in patients with other disorders. Antibodies against TGc might accompany and promote autoimmune diseases, allowing their use in the prognosis of disease activity. Further, the presence of such antibodies in sera from patients with autoimmune disease should not alone be taken as the basis for the diagnosis of coeliac disease or dermatitis herpetiformis.

083

Analysis of Vascular Immunoglobulin and C3 Deposition in Skin of 116 Dermatitis Herpetiformis Patients

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Previously, we had observed vascular immunoglobulin and complement deposition in the skin of several dermatitis herpetiformis (DH) patients. Therefore, we decided to study the significance of this finding. We investigated skin biopsy samples taken from 116 patients with DH. In all cases the diagnosis of DH was confirmed with the presence of granular IgA staining in the papillary dermis. In 40 out of 116 patients studied with direct immunofluorescence we found a significant vascular staining accompanying the DH specific IgA fluorescence. The distribution of staining pattern using antihuman IgA, IgM, and C3 antibodies was the following: IgA vascular positivity in 12 patients, IgM in 9 patients, C3 in 7 cases. Both IgA and C3 positivity was detected in 6, IgM and C3 in 4, IgM and IgA in 2 skin samples. We think that this is a significant finding, which is not a specific symptom in DH, but can help us to understand the pathomechanism of the disease.

084

The Identification of Mutations in Keratin 9 Using Denaturing High Performance Liquid Chromatography

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Epidermolytic palmoplantar keratoderma (EPPK) is characterised by diffuse keratoderma of palm and sole skin and is caused by mutations keratin 9 (K9). We have investigated the use of denaturing high performance liquid chromatography (dHPLC) to screen patients with palmoplantar keratoderma for K9 mutations. DNA from affected individuals from three families and 56 unrelated control samples were analysed for sequence variations in exon 1 of K9. PCR products encoding this region were subjected to heteroduplex analysis by dHPLC using the WAVE™ system. Identified heterozygotic variations were confirmed by DNA sequencing. All samples from affected individuals resulted in complex dHPLC profiles which varied between families. Control samples resolved as either a single peak or a distinct multipeak profile. DNA sequencing confirmed different mutations in each family. In two cases previously reported mutations were identified which resulted in the change of arginine162 to glutamine or tryptophan. In the third case a novel mutation was found which changed valine170 to methionine. The multipeaked dHPLC profile generated by control DNA resulted from a noncoding polymorphism in codon 142 (A426C). The identification of K9 mutations confirmed the diagnosis of EPPK in all three families. dHPLC resolved each heterozygous sequence variation as a different profile and provides a rapid and accurate method of screening for pathogenic and polymorphic DNA sequence variations.

085

Analysis of X-Chromosome Inactivation in Normal Human Epidermis and in Basal Cell Cancer

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During early development of the female embryo, one X-chromosome is randomly inactivated in each cell. As a result of growth, migration and differentiation, the adult female becomes a mosaic of cells with either the paternal or the maternal X-chromosome inactivated. It is not known what structure the X-chromosome inactivation pattern has in skin of normal individuals. We investigated normal skin from four healthy females, heterozygous for the HUMARA microsatellite on the X-chromosome. Following careful microdissection, DNA from adjacent epidermal samples consisting of approximately 35 basal keratinocytes was digested with the methylation-sensitive enzyme HpaII. The inactivated X-chromosome remained intact due to extensive methylation. The enzyme digested DNA was amplified using PCR and fragments were analyzed for size. Through examination of adjacent samples and consecutive sections, we found normal human skin to be composed of a fine mosaic of tiles with either maternal or paternal X-chromosome inactivated. The sizes of these tiles were between 20 and 350 basal cells in diameter. The described method has a potential to resolve the clonal status of normal as well as pathological conditions, and provide important information on early carcinogenesis. We are currently analyzing basal cell cancer, a tumor dependent on a specific connective tissue stroma. Previous studies of tumors with a conspicious stroma component, including biphasic tumors have revealed synovial sarcoma to be a true biphasic tumor whereas phylloides tumor of the breast showed a clonal pattern in the stromal component but not in the epithelial compartment. Preliminary results have uncovered that a subset of basal cell cancers exhibit a polyclonal epithelial component, whereas the stroma appears to be of polyclonal origin regardless of clonal status of the epithelial part of the tumor (unpublished data).

086

A Polymorphism in the PTCH Tumor Suppressor Gene may be Responsible for Multiple Basal Cell Cancers

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We report a polymorphism at codon 1315 in the *PTCH* gene, a tumor suppressor gene that plays an important role in the pathogenesis of basal cell cancer (BCC), and show that this polymorphic locus may affect succeptability for BCC. In DNA of nontumor origin obtained from patients with multiple BCC's, we found an allelic variant of the originally published sequence in exon 22 of *PTCH* where Pro (CCC) occurs instead of the Leu (CTC), which has previously been published as wild-type. In a large set of DNA samples from patients with different genetic background and history for cutaneous malignancy, we analyzed the allele pattern of this codon to find that patients with several BCC's differ in the allele distribution from the control group by having a lower frequency of the Leucine allele. The fact that this polymorphism leads to an amino acid substitution between two amino acids whose chemical characteristics greatly differs further supports our notion. In addition, we also studied allelic loss from tumor DNA of patients heterozygous for this allele and found that a selective loss of alleles can be found in certain patients.

087

Primary Epidermal Cells from Porokeratosis-Affected Patients are not Hypersensitive to UVB or X Irradiation

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The porokeratosis (PK) are a group of skin disorders characterized by the presence of hyperkeratotic plaques which tend to occur at sun-exposed sites. In the case of disseminated superficial actinic porokeratosis (DSAP) a modest hypersensitivity to X irradiation of primary fibroblasts from affected subjects has been reported. In this study we have compared the response to X-rays and UVB radiation of primary keratinocytes and fibroblasts from normal and DSAP-affected subjects. No difference in radiosensitivity was detected when the clonal cell survival of lesional and nonlesional cells from DSAP-affected patients was compared with that of their normal counterpart (cultures from age-matched controls). In general, epidermal keratinocytes were X- and UVB-radiation resistant when compared with fibroblasts. As in the case of radiosensitivity, the response to apoptosis induced by X-rays and UVB was similar in both lesional and non lesional DSAP keratinocytes and in the age-matched controls. Interestingly, primary fibroblasts were refractory to apoptosis in response to either X-rays or UVB treatment while keratinocytes underwent apoptosis after exposure to UVB radiation but not after X-ray exposure. These data clearly indicate that the actinic character of this skin pathology is not due to hypersensitivity to either ionizing or UVB radiation. Mutations in the p53 gene, which are often associated with UV-related skin carcinogenesis, were analysed in a set of porokeratosis lesions. P53 mutations were rarely detected (2/15 lesions examined) and were not UV-specific, confirming that exposure to solar radiation does not play a key role in the etiology of porokeratosis. Preliminary data suggest that premature senescence of keratinocytes might be involved in the development of porokeratotic lesions.

088

Comparison of Gene Expression Between Basal Cell Carcinomas and Squamous Cell Carcinomas

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Basal cell carcinomas (BCC) and squamous carcinomas (SCC) are distinct epithelial skin cancers arising from keratinocytes. BCC grow slowly, nearly never metastases, whereas SCC are invasive tumors with a greater metastatic potential. Some molecular clues for these pathologies have been described (for example alterations in the sonic hedgehog signalling pathway in BCC). In order to investigate other molecular events in the etiology and differential invasiveness of skin carcinomas, we compared large scale gene expression of 7 BCC and 5 SCC with the use of Human Atlas Cancer cDNA expression array (1176 genes, Clontech). For each patient RNA was extracted from tumor and non tumoral control skin. (33)P-labeled cDNA probes were synthesized with Clontech specific primers. The hybridation signal was acquired with a Storm phosphorimager and quantified using the ImageQuant software (Amersham Pharmacia Biotech). Differential expression between tumor and control skin was considered when the ratio of normalized data was above 2 or under 0.5. Among the 1176 genes, 207 genes ± 64 (21%) were differentially expressed between BCC and control skin. 326 genes ± 86 (38%) were differentially expressed between SCC and control skin. 49 similar genes were modulated in 5/7 BCC and 149 in 4/5 SCC. 24 identical genes were modulated in both 5/7 BCC and 4/5 SCC. These included genes involved in cell proliferation, differentiation and interaction with extracellular matrix. Only one gene was specifically overexpressed in BCC: the GEM GTPase gene (implicated in cellular response to growth stimulation). It was 5 fold increased in BCC whereas not modulated or detected in all the studied SCC. 19 genes were specifically modulated in SCC including rhoC and genes implicated in cell-cycle regulation, apoptosis, cellular adhesion and matrix remodeling. Some of these genes may potentially be involved in invasive and metastatic phenotype. In conclusion, this study shows that gene expression in skin carcinomas is vastly modified in comparison with non tumoral skin. Comparison analysis between SCC and BCC shows common and specific gene modulations which are currently explored. Specifically expressed genes could represent new molecular keys for tumor development and local agressivity.

089

Molecular Basis of Skin Cancer: Large Scale Analysis of Differentially Gene Expression in BCC and SCC

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Basal cell carcinoma (BCC) represents the most common malignant neoplasm in humans. BCC occurs primarily in sun exposed areas of the skin. This relationship between sun exposure and carcinogenesis of BCC is based on mutagenic and immunosuppressive effects of the UV range in sunlight. Manifested, UVB induced mutations of DNA could lead to a not physiological expression of cellular genes and altered proteins. Thus, the phenotype of the malignant keratinocyte is expected to be associated with an altered expression profile compared to the normal counterpart of the tumor cell. The aim of this study is to detect differentially expressed genes involved in the pathophysiology of BCC. Here we used human cDNA expression arrays containing 588 cDNA sequences of known genes immobilized on nylon membranes. The membranes were differentially hybridized with labeled cDNA-libraries of pooled BCC- or normal skin samples (NS). In order to investigate the detected differentially gene expression in individual samples we performed semiquantitative RT-PCR using 10 BCC-, 2 SCC-, 11 NS- specimens, HaCaT and normal human keratinocytes (NHK). From the 588 arrayed genes we identified 100 expressed in human keratinocytes. Comparing the expression patterns of the arrayed cDNAs revealed 18 differentially expressed genes, from which 6 were down- and 12 were up-regulated in BCC. The majority of these dysregulated genes (9) are associated with transcription- and cell cycle- control. 4 differentially expressed genes take part in the immunological response, 3 genes belong to a functional group of detoxifying and antioxidizing enzymes and two genes are coding for proteins involved in cell matrix or cell-cell contact.

090

No Evidence of Deregulated Patched-Hedgehog Signaling Pathway in Trichoblastomas and other Tumors Arising within Nevus Sebaceous

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Nevus sebaceous (NS) is a congenital malformation of the skin within which a number of neoplasms showing adnexal differentiation may arise. Recently, deletions in the patched (PTCH) gene region were reported in NS and constitutive activation of the PTCH-hedgehog signaling pathway was implicated in the development of tumors arising within NS. To further substantiate a role of PTCH hedgehog signaling pathway in secondary tumors arising within NS, we examined 11 NS associated with secondary tumors for loss of heterozygosity (LOH) of the PTCH gene region by microsatellite PCR and PTCH mRNA expression by *in situ* hybridization. Unexpectedly, however, none of the tumors (including 8 trichoblastomas) and NS lesions showed LOH at any polymorphic loci close to the PTCH gene. Furthermore, none of the NS lesions and secondary tumors gave detectable signals for PTCH mRNA. In contrast, 4 of 11 sporadic BCCs, that were examined for comparison, showed LOH at the PTCH gene locus ($p < 0.05$), and moderate to strong signals for PTCH mRNA was observed in all 7 BCC tumors examined ($p < 0.0001$). The findings in the present study do not support the view that the deregulation of the PTCH-hedgehog signaling pathway is involved in the pathogenesis of NS and associated tumors, and show that, although morphologically similar, tricoblastomas and BCCs have different molecular pathogenesis.

091

Withdrawn

092

Lamin Expression in Normal Skin, Actinic Keratosis, Squamous Cell Carcinoma and Basal Cell Carcinoma

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Aberant expression patterns of lamins have been described in various types of cancer, and the expression is dependent on the subtype of cancer, its aggressiveness, proliferative capacity and degree of differentiation. This study aims at establishing the expression patterns of lamins in normal human skin, actinic keratosis (AK), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). Expression patterns of the individual lamin subtypes were immunohistochemically studied. In normal skin lamin A yielded positive staining in the suprabasal cell compartment of the epidermis, whereas the basal cells were mostly unstained. The BCCs and SCCs stained practically homogeneously positive, and the epidermis overlying BCC and SCC stained practically homogeneously positive in the basal cells and remarkably strong in the suprabasal cells. In AK, the basal cell layer of the epidermis stained homogeneously positive for lamin A, and the suprabasal compartment showed strongly positive staining. Lamin C was only occasionally expressed in the basal cells of normal epidermis, whereas the suprabasal cells stained strongly positive. In BCCs, lamin C was predominatly present as nucleolar structures, only occasionally was staining of the nucleolar envelop seen. The SCCs stained strongly positive for lamin C, and the staining was predominantly ring shaped. The epidermis overlying BCC and SCC stained strongly positive. The expression of lamin C in AK in the basal cells resembled the expression pattern seen in the epidermis overlying BCC and SCC, but in the suprabasal compartment the staining showed a nucleolar staining. Lamin B1 and B2 stained all cell types in normal skin. The BCCs, SCCs and the epidermis overlying BCC stained strongly positive. The epidermis overlying SCC showed only occasionally staining for lamin B1 in both the basal and the suprabasal cells, whereas lamin B2 was homogeneously expressed in the basal cells and strongly expressed in the suprabasal cells. AK also stained strongly positive for both lamin B1 and B2. We can conclude that there is an abnormal expression of lamins in both premalignant and malignant lesions. Furthermore, the lamin A expression in the basal cell layer of the epidermis overlying BCC may suggest involvement in the primary process.

093

Somatic Mutation of hMLH1 in a Primary Skin Melanoma

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Mutations in the mismatch repair system (MRS) genes have been identified in hereditary nonpolyposis colorectal cancer syndrome and in a low percentage of sporadic tumors presenting high levels of microsatellite instability (MSI). While MSI has been reported in a variable proportion of cutaneous melanomas, no mutations in MRS genes have been described so far in this tumor. We identified a human melanoma cell line, PR-Mel, which was defective in mismatch repair, did not express the MRS proteins hMLH1 and PMS2 and showed high levels of MSI. The cell line was then analyzed for genetic defects in the *hMLH1* gene using an RNA-based screening approach on total RNA. RT-PCR encompassing the cDNA region containing exons 14–18, showed a single aberrant band corresponding to a shorter transcript lacking exon 16. Sequence analysis at genomic DNA level revealed the presence of a G to A transition at position −1 of the acceptor splice site of intron 15, in keeping with exon 16-skipping. The somatic mutation, named 1732–1G→A, was also detected in genomic DNA from both the primary skin melanoma and the cutaneous metastasis from which the PR-Mel cell line had been established. Immunohistochemistry confirmed the absence of hMLH1 and PMS2 expression in the tumor specimens. Both the cell line and tumor samples appeared homozygous for the mutation, suggesting that the normal allele was lost. Indeed, cytogenetic analysis of PR-Mel cells revealed a 3p deletion possibly including the *hMLH1* gene. The identification of the novel mutation 1732–1G→A expands the repertoire of *hMLH1* mutations clustered in the region spanning exons 15 and 16. Moreover, the finding of somatic *hMLH1* genetic defects in a primary skin melanoma suggests that inactivation of MRS genes may be involved in the development of at least some cases of sporadic melanoma.

094

Expression of p16 in Relapsing and Nonrelapsing Very Thin Melanoma

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There is now good evidence that mutation or loss of the tumour suppressor gene p16 is an important factor in the pathogenesis of familial and sporadic melanoma (MM). Most patients with very thin MM (Breslow thickness less than 0.76 mm) are cured by excision however, 2–10% relapse with local or metastatic disease. Previous studies have shown lack of expression of p16 in relapsing MM but there have been no specific studies looking at very thin MM. We have therefore studied the expression of p16 in relapsing and nonrelapsing very thin MM. 18 patients (M:F 7 : 11, median age 47 (28–63) years) were identified with very thin relapsing MM (median Breslow thickness 0.56 mm) and compared with 28 appropriately matched controls (M:F 8 : 20, median age 44 (23–84) years: median Breslow thickness 0.5 mm) who had been followed up for at least 5 years. Immunohistochemistry was performed on formalin fixed sections with antip16 antibody. Tumours were scored for intensity of immune reactivity (IR) on a scale from 0 (no or weak IR) to 4 (very intense IR). Also positive nuclei were counted in at least 5 high power fields ($150 \times 150\,\mu m$) randomly selected throughout each representative tumour. Overall the expression of p16 was less in the patient group than the control group: p16 IR (patients, median 1.5, IQR 0.83–1.95; controls 2.4, 1.5–3); number of positive nuclei (patients 33.5%, 19–40.5; controls 44.5%, 33.1–55.1). However the differences were not significant (Kruskal–Wallis). This study suggests that the expression of p16 is reduced in patients with relapsing very thin MM but does not prove a significant correlation between tumorigenicity and loss of p16 IR.

095

Peptides Binding to MIA (Melanoma Inhibitory Activity) Inhibit MIA Function *In Vitro*

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Analysing the function of MIA, a protein strongly expressed in melanoma cells but not in melanocytes, we determined strong inhibition of attachment by MIA plating melanoma cells onto fibronectin, laminin and tenascin. No inhibition of attachment to collagen type I, II and IV, HSPG and vitronectin was observed. In *in vivo* assays we further showed that MIA plays an important role in melanoma metastasis. Recently, we could solve the 3-dimensional structure of MIA using NMR techniques. MIA adopts a SH3 domain-like fold with two disulfide bridges stabilizing the protein. MIA is the first extracellular protein found to have a SH3 domain fold. A phage display experiment generated proline rich heptapeptide sequences, typical for SH3 domain ligands, which were shown by NMR and ELISA assays to bind to human MIA. In invasion assays using the Boyden Chamber model two of those peptides inhibited MIA action in a dose-dependent manner. Further studies have to evaluate if blocking MIA with these peptides *in vivo* can be used in melanoma therapy.

096

The Inhibitory Effect of Vitamin D3 and Retinoids on the Selected Mechanisms of Tumor Invasion

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Vitamin A and D derivatives were shown to exert various antitumor effects, both in experimental and clinical studies. The aim of the study was to evaluate the effect of 9-*cis* retinoic acid, all-*trans* retinoic acid and calcitriol (1,25(OH)2D3) on the invasive potential of SKv (human cancer cells derived from bowenoid papulosis lesion, found to harbor human papillomavirus 16 DNA) and L1 (murine sarcoma) tumor cell lines. We studied the adherence of the tumor cells preincubated with the examined compounds to culture plates covered with various extracellular matrix (ECM) components, including collagen I, collagen IV, fibronectin and laminin. The attached cells were stained with cristal violet and their number was measured colorimetrically. We also determined the activity of matrix metalloproteinases (MMPs) in the supernatants from the tumor cells incubated with the tested compounds. The proteins of the culture medium were separated electrophoretically on the polyacrylamide gels containing 0.1% gelatin. After the 24 h-incubation the gels were stained with Coomassie blue to visualise the gelatinolytic bands. The preincubation of tumor cells with retinoids at the concentration of 10^{-7} M or calciriol 10^{-6}–10^{-8} M, significantly inhibited their adhesion to the ECM components, especially to collagen I and IV. In case of calcitriol this effect was dose-dependent. In the gelatin zymography we observed the down-regulation of the MMPs activity in the supernatants from the cell cultures incubated with examined agents. The observed inhibitory effects of calcitriol and retinoids on tumor cell adhesion to ECM components as well as MMPs activity may contribute to the antineoplastic potential of retinoids and vitamin D derivatives.

097

CD4+ T-Cells Activated by the Polyaromatic Hydrocarbon DMBA Augment its Carcinogenic Activity in Skin

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The objective of this study was to characterize the T-cells that are activated following topical application of polyaromatic hydrocarbons (PAH) and to determine whether they participate in PAH-induced skin tumorigenesis. Application of the carcinogenic PAH, 7,12-dimethylbenz(a)anthracene (DMBA) to the skin of C3H/HeN mice resulted in the development of contact hypersensitivity to that agent. *In vivo* administration of anti-CD8 antibodies prior to sensitization abrogated the response, whereas *in vivo* depletion with anti-CD4 antibodies augmented DMBA contact hypersensitivity. Treatment of $CD4^+$ knockout mice on a C3H background with DMBA was also found to increase DMBA contact hypersensitivity. When ELISAs were performed on T-cells from DMBA treated mice, $CD8^+$ T-cells were found to produce interferon-γ and little or no IL-4, whereas $CD4^+$ T-cells were found to elaborate both IL-4 and interferon-γ. To determine if $CD4^+$ T-cells participated in PAH skin tumorigenesis, $CD4^+$ knockout and wild type mice were subjected to a DMBA skin tumorigenesis protocol. $CD4^+$ knockout mice developed 80% fewer tumors than wild type mice. The percent of mice with tumors and the number of tumors/tumor bearing mice were also decreased in $CD4^+$ knockout mice. Immunopreventive strategies which suppress the activation of $CD4^+$ T-cells may be successful in reducing PAH skin cancer development.

098

Reduction of Tumor Formation in Scid Mice Xenotransplanted with Human Melanoma Cell Line by Cimetidine and a Tamoxifene Derivate

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Histamine is produced by many cells expressing histidine decarboxylase (HDC), an enzyme responsible for its synthesis. Since melanoma cells and tissue contain relatively large amount of histamine, the functional significance of this finding was examined by specific antihistamines among *in vitro* using human melanoma cell line HT168 and *in vivo* by inoculating human melanoma cell line HT168 to immunodeficient (SCID) mice. Our data show that H2 receptor antagonist cimetidine if combined with a tamoxifene derivate, DPPE (N,N-diethyl-2-{4-(phenylmethyl)phenoxy}-ethanamine-HCl), in addition to their capacity for inhibition of *in vitro* proliferation a strong reduction of melanoma tumour mass is seen *in vivo* with increased survival of the recipient mice. These changes are accompanied by enhanced infiltration of interferon producing mouse macrophages into the tumor tissue of human origin. We suggest, that probably two different mechanisms are acting concordantly, direct proliferation inhibition of the tumor cells by an H2 receptor antagonist and activation of a nonspecific local immune response, characterized by interferon γ production. These findings may help to elucidate the possibility of involvement of a designed antihistamine strategy in melanoma therapy.

099

Microarray and Comparative Genomic Hybridisation Studies of Primary Cutaneous Lymphomas

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Primary cutaneous lymphomas (PCL) represent a heterogeneous group of T- and B-cell neoplasms primarily involving the skin. Little is known, however, about the pathogenesis of PCL. To address this issue we have investigated 31 cases of primary cutaneous T-cell lymphoma (CTCL) and 33 cases of primary cutaneous B-cell lymphoma (CBCL) using microarray and comparative genomic hybridisation (CGH) techniques. We observed chromosome imbalances (CI) in 22 of 31 CTCL cases (71%) and in 20 of 33 CBCL cases (61%) using CGH. The most frequent DNA copy number changes seen in CTCL were losses of 1p (39%), 17p (19%), 10q (16%) and gains of 17q (29%) and 4 (26%). While the most common CI noted in CBCL were gains of 18q/18 (60%), 7p/7 (40%), 4q (20%), 20 (15%), 21q (15%) and loss of 6q (15%). By using microarray based genomic analysis technique, we detected gains of copy number of the oncogenes *PAK1* in 6 of 7 CTCL cases, *RAF1* and *JUNB1* in 5 cases, *CTSB*, *FGFR*, *ZNF* and *BCR* in 4 cases, and loss of *FGR* in 2 cases. We identified gains of *SAS* in 3 of 4 CBCL cases, *BCL2* in 2 case, and loss of *ESR* in 2 cases. Increased copy number of *bcl2* was also confirmed in 4 CBCL cases using real-time PCR. In conclusion, we have identified different patterns of recurrent genetic abnormalities and amplification of oncogenes in CTCL and CBCL. These findings provide the basis for further investigation of the molecular pathogenesis of PCL.

100

Deletions on the Long Arm of Chromosome 6 in Primary Cutaneous B-Cell Lymphoma

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Abnormalities of the long arm of chromosome 6 (6q) involving 6q16–27 have been reported in a variety of malignancies including non-Hodgkin's lymphoma (NHL). Earlier studies using either FISH and/or loss of heterozygosity (LOH) techniques in systemic NHL, and B-CLL have identified 3 regions of minimal deletion (at 6q21, 6q23, and 6q25–27). These regions of allelic loss may indicate the sites of specific tumour suppressor genes (TSG's) whose inactivation may be responsible for tumorigenesis or disease progression. The aim of our study was to identify regions of chromosomal loss on 6q in 35 cases of primary cutaneous B-cell lymphoma (PCBCL) using LOH techniques. Genomic DNA extracted from both tumour and control samples from each patient (a dominant clonal immunoglobulin heavy chain gene rearrangement in tumour, but not control samples) was amplified using PCR and 17 microsatellite markers for the region 6q15–27. Radiolabelled PCR products were separated by 6% denaturing PAGE and analysed by autoradiography. LOH was identified in 3/7 high-grade cases (region of minimal deletion 6q16–21 between D6S434, and D6S447), and 1/28 with low-grade disease at 6q21 (D6S302). Our data suggest that 6q loss may be important in high-grade (region of deletion 6q16–21) but not in low-grade PCBCL. Microdissection to ensure that tumour DNA was highly represented, would help confirm this conclusion. Possible TSG's include CCNC (involved in cell cycle procession), BLIMP-1 (involved in B-cell differentiation), and CD24 (encodes a leukocyte surface antigen). Further mapping of this region is required to define the region of allelic loss more accurately, and may increase the rate of 6q loss in both high, and low-grade PCBCL.

101

Chromosome Aberrations Associate with Progression or Remission in Cutaneous T-Cell Lymphoma

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To evaluate the use of chromosomal abnormalities in clinical follow-up we studied the associations between peripheral blood clonal or nonclonal chromosomal abnormalities and the clinical activity of cutaneous T-cell lymphoma (CTCL) or large plaque parapsoriasis (Pps). Five patients with Pps, 8 with mycosis fungoides and two with Sézary syndrome were followed for the average of 54 months. Two to 4 blood samples of each patient were analyzed with G-banding and enzyme-detected *in situ*-hybridization (EDISH) for chromosomes 1,6,8,9,11,13,15 and 17, previously shown to be frequently aberrated in CTCL. The method allows the use of two probes simultaneously, and gives archivable preparations. Statistically, patients in different diagnostic groups, patients with CTCL, all patients together, and patients with active disease, differed from healthy or PUVA-treated controls for all chromosomes studied with EDISH or G-banding ($p < 0.01$ to $p < 0.05$). Patients in remission differed from healthy controls for chromosomes 1, 6, and 11 ($p < 0.01$ to $p < 0.05$, EDISH). Patients with active, progressing disease differed from patients in remission for aberrations of chromosomes 1,6,8,11 and 17. All 11 samples representing active, progressing disease showed elevated level of chromosome 8 aberrations in EDISH, but so did also 34% of samples representing nonprogressive disease. The agreement rate of both the change in chromosomal aberration rate and in clinical condition between two samples was statistically significant for chromosomes 1,8,9,15 (G-banding) and 17 (G-banding and EDISH; $6 > 0.5$–0.6). Six of seven patients (5 CTCL, 1 Pp patient) with a chromosomal clone in G-banding showed a continuously active disease. Four of them, but no other patients, died within two and a half year from the detection of the clone. Thus, chromosomal aberrations associate with the activity of CTCL, and have prognostic significance. In EDISH, several probes should be used in the follow-up. Aberrations of chromosomes 1,6 and 11, although increasing with activity of the disease, seem to be a hallmark of an existing disease, detected even in remission. Aberrations of chromosomes 8 and 17 associate especially with active or progressive disease

102

NFkB Gene Family Member Expression in CTCL Skin Lesions and IKB Downregulation in CTCL Cell Lines

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The NFkB gene family codes for a series of important transcription factors. Normally NFkB proteins translocate from the cytoplasm to the nucleus only after induction, however, constitutively activated NFkB proteins could lead to aberrant gene expression and cell transformation. Here we investigated whether NFkB proteins are constitutively present in the nuclei of CTCL cells, and whether the constitutive NFkB activities are due to decreased concentrations of NFkB inhibiting IkB proteins. The DNA binding of NFkB proteins was studied by electrophoretic mobility shift assays (EMSA). To determine the expression and localization of NFkB proteins we performed immunohistochemical stainings with antibodies directed against the proteins p50, p52, p65, RelB, c-Rel and Bcl-3. The expression of IkB proteins in CTCL cell lines was investigated by Western blotting. The EMSA and Western blot experiments show, that all 6 tested NFkB proteins are present in the nuclei of the three tested CTCL cell lines, and that at least three of them bind to DNA. Nuclear Bcl-3 and p52 was found in lesions of almost all patients (11/11 and 10/11, respectively). Nuclear p50, p65 and RelB was found in approximately 50% of the patients (5–6/11), nuclear c-Rel only in 3 of 11 patients. The nuclear localization of the latter 4 proteins was restricted to more advanced stages of the disease. In the CTCL cell lines we found that the levels of IkB α and IkB β were strongly reduced in comparison to other cell lines. Our results indicate that reduced IkB levels may be reponsible for the constitutive NFkB activities in CTCL cells and that constitutive NFkB activities also occur *in vivo*.

103

Determination of the Localization of Tryptophan Hydroxylase (TrH)-Immunoreactive (IR) Cells in Normal Healthy Human Skin

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This study has investigated the immunohistochemical localization of TrH, which is the initial and rate-limiting enzyme in the serotonin biosynthesis and also involved in the first nonrate-limiting step in the biosynthesis of melatonin, in normal healthy human skin. Thus, the aim was to find out what kind of cells that have the potential to produce serotonin (5-HT) locally in the skin. Immunofluorescence single-staining of TrH was used to determine the localization of TrH, and double-staining of TrH and the human histocompatibility complex class?subregion DR (HLA-DR), as well as TrH and the melanoma-associated antigen (NKI-beteb), was used to explore the presumed TrH-IR cell types. HLA-DR and NKI-beteb are the commonly used markers for Langerhans cells and melanocytes, respectively. The results show that TrH-IR cells are identical to Langerhans cells (LC) in the epidermis, to LC around the sebaceous glands and vessels of the dermis, and to melanocytes in the epidermal stratum basale. Possibly also some few Merkel cells in the stratum basale, according to their cellular morphology, could be TrH-IR. We conclude that Langerhans cells, melanocytes and possibly some few Merkel cells in the normal healthy human skin have the potential to locally produce 5-HT.

104

Adam 9 Expression in Normal Skin, Wound Healing and Carcinoma

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ADAM 9 is a cell surface protease involved in the shedding of molecules such as HB-EGF. Besides its role as metalloproteinase, ADAM 9 is also thought to mediate cell–cell interactions. Therefore, regulation of ADAM 9 expression might represent an important control mechanism for the release of growth factors and for cell migration. Recent reports have indicated a possible role of PACSINs (3 isoforms of cytoplasmic phosphoproteins involved in vesicular trafficking) in the regulation of ADAMs function. The aim of this study was to analyze expression of ADAM 9 and PACSINs in skin during physiological and pathological processes as wound healing and tumor progression. A polyclonal antibody was used to detect ADAM 9 in cryosections of adult human and mouse skin. In normal skin ADAM 9 expressing cells were localized in the basal layer of the epidermis. Due to the importance of basal keratinocyte during re-epithelialization of skin wounds, we analyzed expression of ADAM 9 during excisional wound repair. At day 3 postinjury, no changes in expression were found in uninvolved skin. However, ADAM 9 was found associated with all keratinocytes at the migrating front. At day 7, when re-epithelialization was almost complete, ADAM 9 expression was reduced in the outer most layers of the epidermis. In human skin tumors, e.g. basal and squamous cell carcinomas there was no ADAM 9 expression detected whereas expression in unaffected skin was unchanged. In normal mouse skin, PACSIN 3 was mainly located in the muscle tissue beneath the dermis. Interestingly, PACSIN 2 was found to be coexpressed with ADAM 9 in basal layers of the epidermis. During wound repair PACSIN 2 expression is also restricted to the basal keratinocytes at the migrating front as well as in the newly formed epidermis. Interestingly, as observed for ADAM 9, PACSIN 2 expression was barely detectable in human skin tumors. These results suggest the involvement of ADAM 9 in the control and maintenance of skin homeostasis. The absence of ADAM 9 expression observed in epidermal tumor cells might indicate a role for ADAM 9 in tumor progression. Finally, this study suggests a putative role for PACSIN 2 in the regulation of ADAM 9 expression.

105

Comparison of Two Types of *Ex Vivo* Models: Hairless Rat Skin on Static Cell and Reconstructed Human Epidermis

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For ethic reasons, alternative methods to animal experiments are needed. Thus in the last past few years, a lot of methods such as reconstructed skin appeared. In order to explore the validity of this new generation of *ex-vivo* models, skin modifications under solar or UVA irradiation were studied by means of two *ex vivo* assays. The first one is constituted by skin samples from hairless rat fixed on a static cell system and maintained with DMEM nutritive medium. The other model is a reconstructed human epidermis model (RHE; Skinethic™) maintained on special growth medium. In both cases, skin samples were irradiated with a Muller reactor equipped with a 450-W xenon lamp, a mirror and a WG 320 mm (1.5 mm) filter for solar irradiation and WG 335 (2 mm) and UG 11 (2 mm) filter for UVA irradiation. The histological modifications of the skin were evaluated after irradiation with doses corresponding to 20 DEM (solar spectra) and 60 J per cm^2 (UVA spectra). The viability of the skin was monitored by the MTT assay. After 24 h, the decrease in viability of skin samples due to experimental conditions was evaluated at around 18%. A solar irradiation gave rise to a decrease in viability of 40.5% for rat skin and is more important for RHE. With an UVA irradiation, an important decrease of the viability was observed and evaluated around 60%.The effect of an irradiation solar or UVA is similar on hairless rat skin maintained on static model and reconstructed human epidermis (skinethic™). The histological studies showed a more drastic effect on the reconstructed human epidermis than on the hairless rat skin, 24 h after the irradiation. The skin displayed an important number of vacuoles, a contraction of the cells in all the epidermis with a separation of the different cell layers. The evaluation of sunburn cells on histological sections, 24 h after the irradiation, showed, respectively, for hairless rat skin and reconstructed human epidermis an average of 1.67 and 1.86 sunburn cells for a solar irradiation, 1 and 1.2 for an UVA irradiation. The sensibility of the reconstructed human epidermis is greater because the number of cell layers is less important than on hairless rat skin. Meanwhile, the two results were similar. In conclusion, these two models were similar in reactivity and data obtained on rat skin samples may be extrapolated.

106

Laser Capture Microdissection: A Potential Tool for Studies on Epidermal Expression of Kallikreins

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The human kallikrein (KLK) gene family which consists of at least 15 serine proteases located at 19q13.4, include the epidermal proteases stratum corneum chymotryptic enzyme (SCCE; KLK7), and stratum corneum tryptic enzyme (SCTE, KLK5). We have previously purified, and characterized these two enzymes, which may both be involved in desquamation, and possibly also in skin inflammation. SCCE and SCTE are produced as zymogens, suggesting they may be part of a proteolytic cascade. This system may include also other kallikreins, since we and others have found that several members of the kallikrein family, in addition to SCCE and SCTE, are also expressed by keratinocyte cell lines and by as yet unidentified cells in whole skin. The aim of the present study was to evaluate Laser Capture Microdissection (LCM) in combination with RT-PCR as a possible tool for elucidating the expression of kallikreins in different parts of human skin. Freeze cut, 6 μm sections of 4 mm punch biopsies of normal skin was microdissected with LCM and three factions were collected: papillary dermis, basal membrane zone, and suprabasal epidermis. From each fraction RNA was prepared and amplified by RT-PCR with primers specific for a number of kallikreins. As markers of separation efficiency we used primers for keratin 14 and keratin 10. Our results show that, the amounts of material obtained from 3 to 6 sections is sufficient for detection of transcripts, also of those occurring in small amounts. However, as compared to most other tissues the superficial parts of skin seems to be more difficult to fractionate with available standard protocols for LCM, possibly due to the inherent cohesiveness of the epidermis.

107

Latent TGF-β Binding Protein LTBP-2 Decreases Cell Adhesion to Fibronectin

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Latent TGF-β binding protein LTBP-2 belongs to the LTBP-fibrillin extracellular matrix protein superfamily. We have analyzed the effects of recombinant LTBP-2 on human fibrosarcoma (HT-1080) and fibroblast cell adhesion using LTBP-2 and its recombinant fragments in quantitative cell adhesion assays as well as morphological studies. Unexpectedly, full length LTBP-2 protein was found to decrease cell adhesion to fibronectin. The antiadhesive effect toward fibronectin was much less prominent than toward collagen type I. LTBP-2 mediated its effects only when present in the growth substratum, not as soluble protein. LTBP-2 had similar although less prominent effects on the adhesion of fibroblasts in quantitative adhesion assays. Although full length LTBP-2 was antiadhesive towards fibronectin, its N-terminal domain alone mediated fibroblast adhesion, but did not augment actin stress fiber formation. The morphology of fibroblasts was changed substantially when plated on substratum coated with fibronectin and LTBP-2 or some of its recombinant fusion protein fragments. Fibroblastic cells cultured on full length LTBP-2 and fibronectin exhibited less adherent morphology showing altered actin cytoskeletons, but they formed extensive membrane ruffles. Unexpectedly, the actin cytoskeleton disturbing effect, displayed by the full-length protein, was localized to the N-terminal adhesive region of LTBP-2. Cells grown on substratum coated with this fragment were less spread when compared to both the cells plated on fibronectin or adhesive fragment alone. The observations suggest coordinated roles for LTBP-2 together with fibronectin in the regulation of cell adhesion, and have implications in the regulation of wound healing.

108

Procyanidin B-2, a Protein Kinase C (PKC) Inhibitor Possessing the Ability to Promote Hair Growth, Modulates the PKC Isozyme Expression in Hair Epithelial Cells

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Procyanidin B-2 [epicatechin-(4β→8)-epicatechin] is a polyphenol compound classified as a proanthocyanidnin, which we have identified in apples which acts as a hair-growing factor in the murine model both *in vitro* and *in vivo*. Procyanidin B-2 is also known as a compound showing selective PKC inhibition. In this study, we investigated the mechanisms of action of hair-growing activity possessed by procyanidin B-2 focusing on its effects on PKC pathway in hair epithelial cells. We examined whether it modulates the expression or translocation of PKC isozymes in hair epithelial cells and whether it modulates the intracellular calcium level in hair epithelial cells. As a result, we observed that procyanidin B-2 reduces the expression of PKC-α, -βI, -βII, and -η in cultured murine hair epithelial cells and also inhibits the translocation of these isozymes to the particulate fraction of hair epithelial cells. Procyanidin B-2 did not affect intracellular calcium level in hair epithelial cells. We speculate that the hair-growing mechanisms of procyanidin B-2 are at least partially related to its down-regulation of PKC isozymes or its inhibition of translocation of PKC isozymes to the particulate fraction of hair epithelial cells, in addition to its selective PKC inhibition.

109

Is Desquamation Regulated by a Serine Protease Cascade?

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We have previously purified and characterized two epidermal proteases, stratum corneum chymotryptic enzyme (SCCE), and stratum corneum tryptic enzyme (SCTE). Both enzymes, which may be involved in desquamation and possibly also in skin inflammation, are produced as zymogens which can be activated by tryptic cleavage. SCCE and SCTE may thus be part of a proteolytic cascade with as yet unidentified activating enzymes. In the present study we addressed the question whether it was possible to produce catalytically active recombinant SCTE in amounts sufficient to allow a further characterization of its catalytic properties, including its potential role as an activating enzyme for pro-SCCE. The cDNA of pro-SCTE was modified in order to replace the native activation site with an enterokinase cleavage sequence and inserted into an insect cell expression vector. The expressed protein was purified by a combination of reversed phase and ionic exchange chromatography, and was shown to be converted to active protease by treatment with enterokinase. The active recombinant enzyme was incubated with recombinant pro-SCCE, and proteolytic activity was analyzed with chromogenic peptide substrates and zymography. Protease activation in the stratum corneum may be an important regulatory mechanism of desquamation. The results showed that recombinant SCTE could convert pro-SCCE to active SCCE at a low but significant rate. The majority of the total SCCE found in the stratum corneum is present as pro-enzyme. This suggests that the activation of SCCE is slow under physiological conditions. We conclude that SCTE can act as an SCCE-activator.

110

Effects of Subtoxic Doses of Aldehydes and/or UV-Irradiation on Primary Human Dermal Fibroblasts *In Vitro* Depend on Culture Conditions

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Aldehydes are ubiquitous found in the environment. Subtoxic conentrations of aldehydes are components of cosmetics, textile finishers, desinfectants, tobacco smoke and chipboards affecting skin and respiratory epithelia. Toxic effects of high doses have been demonstrated. Effects of subtoxic aldehyde doses (below MAK-value; maximal workplace concentration: 600 µg per m^3 per 8 h) and in addition to other subtoxic stresses, e.g. UV-irradiation were not tested yet. Subtoxic concentrations of acetaldehyde, formaldehyde, and acrolein (10 µM), exposed for 8 h, with/without UV-irradiation (15 mJ per cm^2 UVB) were evaluated using primary human dermal fibroblasts under serum-free and serum-supplemented culture conditions. Expression of MIB 1, p53, involucrin, HSP 72 and HSP 27 were examined immunocytochemically. Proliferation was quantified fluorometrically 24, 48, and 72 h after aldehyde-exposure using the AlamarBlue assay. Aldehyde-induced DNA-damage was evaluated by COMET-assay. None of the aldehydes and UV-irradiation tested induced DNA-damage or influenced p53-expression. Involucrine-expression was slightly elevated by all aldehydes. Acetaldehyde slightly up-regulated HSP 27- and HSP 72-, and increased MIB 1- expression. Formaldehyde slightly increased HSP 27-expression, but highly HSP 72-expression. Acroleine highly up-regulated HSP 27 and HSP 72 but inhibited MIB 1. 24 h after coexposure to aldehydes and UV-irradiation formaldehyde induced a higher proliferation rate than acetaldehyde under serum-free conditions, which was opposite under serum-supplemented conditions. Up to 72 h after coexposure to aldehydes and UV-irradiation proliferation was reduced under serum-free but elevated under serum-supplemented conditions, compared to controls. A subtoxic concentration of aldehydes had evident effects on cultured cells. Co-exposure to aldehydes and UV-irradiation reduced viability of cells in culture. Effects of aldehydes on protein-expression and proliferation depended on culture conditions used. Optimised cell culture conditions are essential for the evaluation of subtoxic aldehyde effects and will be used for the investigation of mediator synthesis, e.g. cytokines and prostanoids.

111

Human Dermal Fibroblasts Escape from the Long-Term Phenocopy of Senescence Induced by Psoralen Photoactivation

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We have previously shown that following psoralen photoactivation (PUVA treatment) human dermal fibroblasts undergo long-term growth arrest as well as morphological and functional changes reminiscent of cellular senescence. In the absence of molecular data what constitutes normal senescence, it has been difficult to decide whether these PUVA-induced changes reflect cellular senescence or rather a mimic thereof. We herein report that PUVA-induced growth arrest, the senescent phenotype and the long-term induction of senescence-associated â-galactosidase and matrix-metalloprotease-1 (MMP-1) are fully reversible at days 100–130 post PUVA treatment in four independently tested fibroblast strains. The late returning growth capacity in PUVA-treated fibroblasts is neither due to transformation nor immortalization as shown by anchorage dependent growth, unchanged telomerase activity, accelerated telomere shortening with increasing cumulative population doublings, and a decrease in overall growth rates (cumulative population doublings) in fibroblasts in their regrowing phase post PUVA treatment. We, furthermore, provide evidence that PUVA-treated fibroblasts retain a memory of the preceding PUVA damage as in their regrowing state they reached the stationary phase of replicative senescence at a much lower cumulative population doubling compared to mock-treated control fibroblasts of the same strain. Oxidative telomere shortening may be involved as a stress sensor responsible for damage recording and limited population dynamics. Collectively, our data suggest that PUVA-induced changes do not fully reflect replicative senescence but rather represent a long-term transient phenocopy thereof. The herein reported model is particularly suited to elucidate mechanisms underlying the long-term transient growth arrest and related functional changes.

112

Blockade of Advanced Glycation End-Products (AGEs)-Receptor for AGEs (RAGE) Interaction Suppress Human Melanoma Cell Growth

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Previous immunological demonstration of AGE in several human tissues suggests that AGE may be involved in aging processes, diabetic complications, and atherosclerosis. In addition, the receptor for advanced glycation end products (RAGE), one of the receptors for AGEs, has been reported to interact with distinct molecules implicated in homeostasis, development and inflammation. Recently it was reported that RAGE regulates the invasiveness, growth, and movement of malignant cells, required for malignancy. In order to investigative the role of AGEs-RAGE in human melanoma, we examined the cell growth effect of AGEs (AGE1-5, CML: carboxymethyllysine) and RAGE in melanoma cells. AGE1–5were able to up-regulate the proliferation rate of G361 cells, human melanoma cell line, dose-dependently. We also demonstrated that the cell proliferation rates of G361 cells were inhibited by anti-RAGE Ab treatment compared to nonimmune IgG treatment of control cells. Immunohistochemical analysis with these antibodies revealed that a strong staining for AGE1-5 and RAGE in melanoma cells. Our results indicate that both AGEs immunoreactivity is highly positive and RAGE is expressed in the human melanoma cells and that AGEs/RAGE interactions promote melanoma cell proliferation *in vitro*. This work opens the way to develop new strategies using modulate AGE–AGE receptor interactions to treat malignant tumors.

113

Ascorbate and α-Tocopherol Decrease in Human Skin During Aging

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Despite increasing evidence that reactive oxygen species play a role in aging, photoaging, and aging associated skin diseases, data about the antioxidant capacity of skin during the aging process are very scarce. For human studies this may be partially due to technical difficulties in measuring low molecular weight antioxidants in skin. We established a refined methodology for the measurement of low molecular weight antioxidants, which allowed us to look for potential changes in ascorbate, α-tocopherol and urate skin concentrations during aging. Furthermore, to account for inherent differences in skin layers which reflect predominantly exogenously exposed and nonexposed skin, frozen skin biopsy punches were horizontally dissected into an upper layer of 1.5 mm and a lower one of 1.5 mm thickness. We found a significant difference between the amount of ascorbate and α-tocopherol present in the upper 1.5 mm skin (epidermis and upper dermis) compared to the lower dermal compartment (lower dermis and parts of subcutis). Ascorbate was seven folds higher in the upper 1.5 mm whereas α-tocopherol had a 6 fold higher concentration in the lower 1.5 mm skin. Ascorbate showed a significant decrease with increasing age in the upper 1.5 mm ($p < 0.05$) and α-tocopherol concentration decreased with increasing age in both compartments ($p < 0.01$ and $p < 0.05$), while urate did not show any correlation with age. A decrease of antioxidants has been described in other organs in humans. The data reported in this study indicate that there is a significant decrease of two major small molecular weight antioxidants in human skin during the aging process. Hence, it seems reasonable to suggest a higher dietary intake of fruits and vegetables for older people as one possibility to increase ascorbate and α-tocopherol levels.

114

Induction of SSP3502 by Sodium Lauryl Sulfate in Human Skin: an Early Marker for Skin Irritation?

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To evaluate the potential toxic hazard for human skin, individual compounds as well as end products are being screened extensively, mainly focusing on acute skin toxicity. Using proteomics, the aim of the present study was a search for markers of which the expression changes after mild irritation. For this, sodium lauryl sulfate (SLS) was applied topically on excised human skin. Epidermal proteins were isolated from SLS-treated skin specimens hardly showing any morphological changes. Subsequently, the proteins were analysed by 2 dimensional polyacrylamide gel electrophoresis (2D-PAGE) and proteins that significantly increased or decreased after SLS-treatment in a dose-dependent way were characterised by mass spectrometry. We identified 7 proteins as potentially new epidermal markers for skin irritation. Among these 7 proteins, SSP3502 was recognized as the most prominently up-regulated protein in all four experiments. Using an antibody raised to SSP3502, a strong staining was seen in the SLS-treated skin, whereas in the vehicle controls only weak staining was observed. Moreover, staining was also observed after topical application of SLS *in vivo* on skin of healthy volunteers. SSP3502 may therefore be used as an early marker of skin irritation and eventually as a novel tool in the clinic allowing testing the sensitivity of patients for a panel of irritants.

115

HSP70-Rich Yeast Extract Offers a New Approach to Skin Care and Treatment

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Scientific data have shown that stress induction of Heat Shock Proteins (Hsp), the widely expressed chaperons, helps cell survival and tolerance to stress. Here we bring evidence that providing the cell with Yeast-Hsp extract, in a stress free manner, gives higher protection to the cell and improves its defense against different stresses. An extract rich with yeast-induced Hsp70 and heat shock factor (which regulates Hsp expression) was prepared by Biotechnology and administered to the cells in these studies. Dose studies were conducted on human fibroblasts during various points over time. Total protein extract as well as total RNA were prepared. Hsp70 expression was examined by immunoblotting. In parallel, Hsp70 mRNA level was studied in Northern blot experiments. Immunoblotting studies demonstrated that fibroblasts treated with the yeast extract-Hsp show a very rapid increase (within 30 min) in their Hsp70 content. The Northern blot experiments revealed a transient and reproducible Hsp70 mRNA expression increase 3–5 h after the incubation with the yeast extract. Supported by the great sequence homology of 70% to 85% with human Hsp, our result suggests that the early increase in Hsp is due to yeast Hsp. Additional studies showed that cells treated with yeast Hsp demonstrate no sign of cell stress and a significant immediate increase in their thermal and UV tolerance. Moreover, this beneficial effect was confirmed by studies on *ex vivo* skin. These interesting results provide the first evidence that Hsp70 extract stress-free administration to the cells enhances their protection and defense from stress.

116

Glucocorticoids Induced the Production and Gene Expression of IL-1?l through NF-êbb and AP-1 Activation in Murine Epidermal Cells

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To investigate the mechanism of the glucocorticoids (GC)-induced augmentation of skin response, we have recently reported the modulatory effect of GC on the regulation of cytokines produced by keratinocytes stimulated with various chemicals *in vitro* (Miyazaki Y et al. JID 115; 746–753, 2000). In that report, we demonstrated that a low concentration of GC augments both NF-êBB and AP-1 activity in the hapten-stimulated Pam 212 cells (Keratinocyte cell line). To clarify the mechanism of the GC-induced augmentation of cytokine production, we examined the modulatory effect of GC on keratinocytes without any stimulation. 10^{-4} M GC inhibited the production of IL-1? from Pam 212 cells; however, 10^{-8}-10^{-10} M GC significantly enhanced the production of IL-1?M by Pam cells at both the protein and mRNA levels. In contrast, GC had no or non significant effect on the production of either TNF-?t, IL-6, IL-10, or GM-CSF by Pam 212 cells incubated for 6 h. Electrophoretic mobility shift assay (EMSA) revealed that 10^{-10}-10^{-12} M GC induced the NF-êBB activation in Pam 212 cells, while 10^{-8}-10^{-10} M augmented the AP-1 signal. Furthermore, pyrrolidinedithiocarbamate (PDTC) partially inhibited the IL-1?F production and completely inhibited NF-êBB activation by Pam 212 cells. On the other hand, MAP-Kinase inhibitors (PD98059 or SB202190) abrogated both the low concentration of GC-induced IL-1?o production and AP-1 activation. These data indicated that the low concentrations of glucocorticoids can induce the augmentation of IL-1?T production from nonstimulated keratinocytes mediated mainly through AP-1 pathway and partially through NF-êBB.

117

Corticotropin Releasing Hormone Receptor mRNA Levels are Regulated by Testosterone, 17β-Estradiol and Growth Hormone in Human Sebocytes

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Hypothalamic-pituitary-adrenal (HPA) axis equivalent pathway has been proposed to function in mammalian skin in response to dyshomeostatic stimuli. Sebaceous glands seem to be involved in this type of response as they express receptors for α-melanocyte-stimulating hormone (α-MSH), vasoactive intestinal polypeptide, neuropeptide Y, and calcitonin gene-related peptide (CGRP). α-MSH and CGRP are not produced by human sebocytes but regulate their cytokine synthesis in a paracrine manner. Moreover, steroid hormones exhibit a dinstinct action on genes related to immune and neuroendocrine regulation. Because corticotropin releasing hormone (CRH) is the most proximal element of the HPA axis we investigated the mRNA levels of CRH and CRH receptors (CRH-Rs) in SZ95 sebocytes and their regulation by several hormones using TaqMan RT-PCR. CRH mRNA was neither expressed nor induced by the hormones tested. In contrast, CRH-R1 and CRH-R2 mRNA were detectable in SZ95 sebocytes at similar levels after 6 h in serum-free culture. CRH-R1 levels significantly increased at 24 h (CRH-R1/CRH-R^2 = 2). 17β-Estradiol stimulated CRH-R1 mRNA levels at 6 h, whereas testosterone at a high concentration (10^{-7} M) annulled CRH-R1 and CRH-R2 mRNA expression at 24 h. Growth hormone (10^{-7} M) annulled CRH-R1 and increased CRH-R2 mRNA levels at 24 h. Dehydroepiandrosterone and insulin growth factor-1 barely influenced CRH-R mRNA levels. All hormones tested did not regulate intrinsic SZ95 sebocyte necrosis. In conclusion, CRH-Rs but not CRH mRNA were detected, indicating that CRH is also a paracrine hormone for human sebocytes. CRH-R1 is the slightly predominant type. The low CRH-R1 mRNA levels in testosterone-treated sebocytes correlate with those found in telogen hair follicles and the high levels in 17β-estrogen-treated sebocytes with those detected in anagen follicles. The switch of predominant CRH-R1 mRNA expression to a sole CRH-R2 expression by growth hormone indicates a possible switch of ligands; growth hormone may facilitate CRH-R binding of sauvagine- and urotensin I-like molecules, instead of CRH. Therefore, CRH-Rs are likely to be involved in sebaceous gland response to dyshomeostatic stimuli and this response may be regulated by several hormones.

118

Sebocytes are the Key Regulators of Androgen Homeostasis in Human Skin

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The mRNA expression patterns of androgen receptor and androgen metabolizing enzymes 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase (Δ^5–3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) were investigated in three different cell populations, SZ95 sebocytes, HaCaT keratinocytes and MeWo melanoma cells by means of RT-PCR. Restriction analysis of cDNA fragments was performed to identify isozymes of Δ^5–3β-HSD and 3α-HSD. In addition, ^{3}H-dihydroepiandrosterone and ^{3}H-testosterone were used as substrates to determine the metabolic activity of these enzymes in SZ95 sebocytes, primary sebocyte cultures and HaCaT keratinocytes. Furthermore, the effects of the selective 5α-reductase type 1 and 2 inhibitors, MK386 and dihydrofinasteride, respectively, and of the Δ^5–3β-HSD inhibitor cyproterone acetate on androgen metabolism were investigated. Androgen receptor mRNA was detected in SZ95 sebocytes and HaCaT keratinocytes but not in MeWo melanoma cells, whereas Δ^5–3β-HSD isotype 1 mRNA and metabolic activity were only found in SZ95 sebocytes. The enzyme activity could be inhibited by cyproterone acetate. Type 2 17β-HSD, type 1 5α-reductase and 3α-HSD mRNA were expressed in all three cell populations tested, while type 3 17β-HSD mRNA could only be detected in SZ95 sebocytes. The major metabolic steps of testosterone in SZ95 sebocytes, primary sebocyte cultures and HaCaT keratinocytes were its conversion to androstenedione by 17β-HSD and further to 5α-androstanedione by 5α-reductase. The type 1 5α-reductase selective inhibitor MK386, but not the type 2 selective inhibitor dihydrofinasteride, inhibited 5α-reductase at low concentrations in SZ95 sebocytes and HaCaT keratinocytes. 5α-Androstanedione was degraded to androsterone by 3α-HSD which exhibited a stronger activity in HaCaT keratinocytes than in SZ95 sebocytes and in primary sebocyte cultures. Lower levels of 5α-dihydrotestosterone and 5α-androstanediol were also detected in all cells tested. In conclusion, specific enzyme expression and activity in sebo-cytes and keratinocytes seem to allocate different duties to these cells *in vitro*: Sebocytes are able to synthesize testosterone from adrenal precursors and to inactivate it in order to maintain androgen homeostasis, while keratinocytes are rather responsible for androgen degradation.

119

Ethanol Stimulates the Proliferation of HaCaT Keratinocytes. The Possible Role of Alcohol in Exacerbating Psoriasis

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Alcohol abuse can precipitate the hyperproliferative skin disease psoriasis vulgaris. The mechanism by which alcohol affects this disease is still elusive. Previously we found that acetone, which exceeds its normal endogenous level in the blood of heavy drinkers, up-regulated the α5 integrin mRNA levels and induced the proliferation of HaCaT cells. In the present study we examined how ethanol affects the proliferation, the IL-8 mRNA production and the mRNA levels of genes characteristic for proliferating keratinocytes such as α5 integrin, keratinocyte growth factor receptor and cyclin D1 of HaCaT cells, a commonly used model to study psoriatic keratinocytes. HaCaT keratinocytes were incubated for 72 h in the presence of different ethanol concentrations (2.1375 μM–1.71 mM) and the number of viable cells was determined by the MTT assay. After HaCaT cells were treated with 4275 μM ethanol concentration the expression of IL-8, α5 integrin, keratinocyte growth factor receptor and cyclin D1 genes was studied by semiquantitative RT-PCR at different time points (0.5 h, 3 h, 6 h). Band intensities of RT-PCR products were quantified by densitometric scanning and values were normalized against the β-actin housekeeping gene. Our data showed that ethanol induced the proliferating potential of HaCaT cells (maximum increase was observed at 4275 μM ethanol concentration) and at all time points α5 integrin, keratinocyte growth factor receptor and cyclin D1 mRNA levels were higher compared to the controls, wheras ethanol did not alter the IL-8 production of HaCaT cells. Our present results suggest that ethanol enhances the proliferation of HaCaT cells and also the expression of different genes supposed to be involved in the pathogenesis of psoriasis. This stimulatory effect of ethanol on human keratinocytes may be one of the reasons why psoriasis can be precipitated by alcohol misuse.

120

Phototoxicity to Diuretics and Antidiabetics in the Cultured Keratinocyte Cell Line HaCaT. Evaluation by Clonogenic Assay and Single Cell Gel Electrophoresis (Comet Assay)

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The oral antidiabetics tolbutamide, glibenclamide, and glipizide, and the diuretics bendroflumethiazide, butizide, furosemide, hydrochlorothiazide, and trichlormethiazide were investigated for their potential to cause phototoxicity in the HaCaT cell line. The cells were incubated with different concentrations of the drugs and then exposed to UVA1 irradiation. Cell survival was evaluated in a clonogenic assay and phototoxic DNA damage was investigated by the single cell gel elctrophoresis (comet assay). The effects of the antioxidants L-ascorbic acid, and a-tocopherol on oxidative DNA damage were also assessed. Bendroflumethiazide, furosemide, hydrochlorothiazide, trichlormethiazide, or tolbutamide induced dose-dependent phototoxicity in the clonogenic assay. Cells incubated with bendroflumethiazide, tolbutamide, and glibenclamide, and irradiated with UVA1 demonstrated an increased oxidative DNA damage revealed as alkali-labile sites in the comet assay. Pre-treatment with L-ascorbic acid, or a-tocopherol, suppressed the UVA-induced DNA damage in cells incubated with 1 mM of bendroflumethiazide, furosemide, glibenclamide, glipizide, tolbutamide, and trichloromethiazide, further implying the involvement of reactive oxygen species in the phototoxic DNA damage. These results indicate a link between phototoxic and photocancerogenic potential of the sulfonamide-derived oral antidiabetic and diuretic drugs, as it has previously been recognized for psoralen, chlorpromazine, and fluoroquinolones. Excessive exposure to UV light may be deleterious for patients treated with oral antidiabetic and diuretic drugs.

121

Stimulation of Water Flux and Epidermal Differentiation on Reconstructed Human Epidermis by an Ajuga Turkestanica Extract

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Aquaporin belongs to a family of water channel proteins involved in water and solute transport particularly in epithelial cells. Epidermis is a stratified and keratinized epithelium, and forms the primary barrier to body water loss. The purpose of this work was to examine the effect of an *Ajuga turkestanica* extract (AT), enriched in iridoids and turkesterone, both on aquaporin mediated water flux and differentiation process of the reconstructed human epidermis (RHE). Using a polyclonal antiaquaporin 3 antibody, Western blot on human epidermis and immunofluorescence on isolated human skin cross section demonstrated the presence of aquaporin 3. This aquaporin was located at the keratinocyte plasma membrane. Functionality of water channel was also examined ($n = 3$) on native stripped and 3 weeks RHE. Osmotically induced water flux (150 mOsm) were comparable, > 50% inhibited by 1 mM $HgCl_2$, a known inhibitor of aquaporin, and fully stopped by acidic pH, pointing out the major role of aquaporins in epidermal water movement. Measurable spontaneous water flux were also present in stripped native skin and RHE. After 17 days RHE treatment at a final concentration of 2.5 µg per mL, AT significantly increased these water flux compared to nontreated controls (0.12 ± 0.03 vs. -0.015 ± 0.048 µL per min per cm^2). Interestingly, treated RHE exhibited a more developed horny layer. Electron microscopy revealed more well differentiated desmosomes, sticker cornified envelope and thinner corneocytes with narrow intercellular spaces and more numerous corneodesmosome structures, compacted and well-oriented keratin network connected to desmosomal plaques. AT appears to be a good candidate for the regulation of skin hydration by stimulating spontaneous water flux, terminal differentiation and cohesion of human epidermis.

122

Permacol™ as a Possible Dermal Substitute for Full Thickness Wounds

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The suitability of Permacol™ as a biocompatible dermal graft to aid the healing of full thickness wounds has been investigated both *in vivo* and *in vitro*. Permacol™ is an acellular porcine-derived dermal collagen implant (CE class III), used clinically for permanent tissue repair. Unlike other collagen implants, it retains it's native fibrous dermal collagen structure, and has been cross-linked to stabilise against enzymatic digestion *in vivo* using hexamethyl- diisocyanate (HMDI). For the *in vivo* study, 0.35 mm thick Permacol™ sheet was grafted into 1 cm^2 full thickness surgical wounds created on the backs of male PVG rats. Wounds were covered with nonadhesive semiocclusive dressing (Opsite), bandaged for up to 21 days and allowed to heal. Care was taken not to allow Permacol™ to dry out at any time. Wounds were inspected, photographed and biopsied at 14 and 28 days, and 3 months postgrafting. Upon inspection, grafts had retained their original proportions, with little or no wound contraction. No overt signs of localised inflammatory reaction were seen. By 14 days, grafts were beginning to integrate into the surrounding tissue, and by 28 days they had become fully re-epithelialised. Histological analysis showed complete integration of grafts at this stage, with recellularisation by dermal fibroblasts, and attachment of normal stratified epidermis. For the *in vitro* study, the ability of Permacol™ to form a three-dimensional skin equivalent has been investigated. For this purpose, Permacol™ pieces were seeded with human dermal fibroblasts and primary epidermal keratinocytes and maintained in culture for up to 28 days. Cultures were fixed for analysis by histology, immunohistochemistry and elecrton microscopy. Results so far have demonstrated proliferation of fibroblasts to confluence, attachment of keratinocytes and *de novo* expression of basement membrane components in these cultures. We believe that Permacol™ has potential as a biocompatible dermal implant to aid the healing of problematic full thickness wounds, either in its original form or more likely as a modifed form specifically for this purpose. Clinical studies to confirm this are currently being conducted.

123

Altered Keratin 16 Gene Expression in Acute and Chronic Wounds Involves the Transcription Factor NF-IL6

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Acute and chronic wounds are characterised by altered keratin expression involving up-regulation of inducible keratins (K6, K16 and K17). These keratins are also induced in other hyperproliferative states, absent in normal epidermis and constitutively expressed in skin appendages (hair follicle, sebaceous gland, and sweat gland). Their transcriptional regulation in terms of tissue-specific gene regulation in not fully understood. In studies examining various transcription factors in normal and diseased epidermis (psoriasis, genodermatoses and wound healing), we have found that NF-IL6 is intimately connected with K16 but not K17 gene expression. We have examined the control of K16 and K17 expression *in vivo* and find that translocation of NFIL-6 to the nucleus of suprabasal cells is coincident with cytoplasmic K16 expression but not K17 expression. Frozen skin biopsies from 10 patients with chronic venous leg ulceration (VLU), psoriatic patients (4) and normal controls (4) were cryostat sectioned and treated with antibodies to K16 or K17 alone, or together with NFIL-6. They were visualised with streptavidin-FITC or antibody conjugated Texas Red. Sections were examined by fluorescence photomicroscopy and confocal microscopy. Control sections with no primary antisera were prepared and some sections were incubated with propidium iodide (nuclear specific stain). NFIL-6 was cytoplasmic in basal cells, nuclear in suprabasal cells and absent from upper granular cells. Nuclear NFIL-6 was present in mid-epidermis of normal skin but dramatically increased in epidermal suprabasal cells at the edge and at the periphery of acute and chronic wounds. Nuclear translocation of NFIL-6 was always accompanied by K16 expression in wounded but not in normal epidermis and was independent of K17 expression. Thus, NFIL-6 is necessary but not sufficient for induction of K16 gene expression and unlikely to be involved in K17 expression.

124

Keratin Expression in Reconstituted Human Skin is Markedly Affected by Retinoids Acting via the Retinoic Acid Receptor α

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Retinoids are used therapeutically in keratinizing disorders but their influence on epidermal keratin expression is poorly understood. *In vivo*, suprabasal keratinocytes normally express K1, K2e and K10, whereas cultured cells also express K4 and K13 which are present in mucous epithelia or appear in the granular layer after topical application of retinoic acid (RA) to normal skin. To learn more about the retinoid-regulation of keratin expression under *in vivo*-like conditions, we studied human keratinocytes grown on de-epidermized dermis in 0.5% serum which produced a normal-looking epidermis that expressed high amounts of K1, K2e and K10 mRNA by real-time PCR, but only minimal amounts of K4 and K13. Addition of RA to the medium for 48 h caused dose-dependent increase in K4/K13 and down-regulation of K2e mRNA. By studying 10 retinoid agonists with different affinities for the RAR and RXR isoforms, the reciprocal effects on K2e and K4/K13 could be connected to the RARα agonists. One of these agonists, Am580, caused a concerted up-regulation of K4/K13 and down-regulation of K2e that was apparent already 8 h after drug addition and that was 10–100 fold already at 10^{-8} M of the retinoid. The effects on K4 and K13 were markedly inhibited by coaddition of a panRAR antagonist (AGN193109), whereas the down-regulation of K2e was less affected. In conclusion, RARα agonists modulate keratin expression, which may be involved in the mechanism of action of retinoids in epidermal diseases.

125

The Catalog of Human Hair Keratins. Expression of the Six Type II Members in the Hair Follicle and the Complete Catalog of Human Hair Keratins

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The human type II hair keratin subfamily consists of six individual members which can be divided into two groups. Group A members hHb1, hHb3, and hHb6 are structurally related, while group C members hHb2, hHb4, and hHb5 are rather distinct. Specific antisera against the individual hair keratins were used to establish the two-dimensional catalog of human type II hair keratins. In this catalog, hHb5 showed up as a series of isoelectric variants, well separated from a lower, more acidic and complex protein streak containing isoelectric variants of hair keratins hHb1, hHb2, hHb3 and hHb6. Both *in situ* hybridization and immunohistochemistry on anagen hair follicles showed that hHb5 and hHb2 defined early stages of hair differentiation in the matrix (hHb5) and cuticle (hHb5, hHb2), respectively. While cuticular differentiation proceeded without expression of further type II hair keratins, cortex cells simultaneously expressed hHb1, hHb3, and hHb6 at an advanced stage of differentiation. In contrast, hHb4, undetectable in hair follicle extracts and sections, could be identified as the largest and most alkaline member of this subfamily in cytoskeletal extracts of dorsal tongue. This hair keratin was localized in the posterior compartment of the tongue filiform papillae. Comparative analysis of type II with the previously published type I hair keratin expression profiles suggested specific, but more likely, also random keratin pairing principles during trichocyte differentiation. Finally, by combining the type I and II hair keratin catalogs with that of cytokeratins, we present the complete catalog of presently known human hair-/cytokeratins.

126

The Tape Stripping of Human Skin: An Approach for Studying Human Epidermal Gene Expression Using Real Time Quantitative RT-PCR

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Biopsies are used for gene expression studies of human skin. Biopsies are invasive and request an ethical approval from an ethics committee. The technique of tape stripping has been reported for studying damaged skin. The purpose of this work was to evaluate an alternative approach to skin biopsies for investigating gene expression of healthy volunteers' epidermis using the tape stripping approach. Firstly, we carried out two series of stripping on the volar forearm skin: 20 strips and after 72 h, 8 strips, in order to collect cells containing a nucleus. Secondly, total RNA extraction was conducted using cells obtained with stripping after 72 h. Finally, after a non specific reverse transcription, a real time quantitative PCR (LightCycler, Roche) was performed on the 26S ribosomal protein cDNA. We were able to detect the sequence for the 26S ribosomal protein indicating that the tape stripping technique could be a noninvasive alternative method for isolating epidermal cells and for studying gene expression at the epidermis level.

127

Identification of Epidermal Stem Cells with a Combination of Flow Cytometry, Cell Culture and Image Processing

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Epidermal keratinocytes can cell kinetically be distinguished into three categories with different functional and morphological characteristics. Stem cells (strong expression of β1-integrin) that have an unlimited clonal potential, transient amplifying cells (weak expression of β1-integrin) with a limited number of cell divisions and terminally differentiated cells (no expression of β1-integrin) that lost the ability to divide. Our final objective was to define these different epidermal subpopulations on the basis of their phenotype and clonal potential. In this investigation, a flow cytometric assessment and cell culture experiments were optimised for selecting potential stem cells. At first we determined which method was optimal to obtain single cell suspensions. With that method we sorted flow cytometrically three subpopulations: cells that were negative, weakly positive (dim cells) and strongly positive (bright cells) for β1-integrin expression. Subsequently, the clonal potential of these three subpopulations was investigated in cell culture experiments. In order to visualise colonies earlier, fluorescent DNA dyes were added. Image processing was used to detect fluorescent colonies and record growth characteristics. Incubation in 0.25% trypsin solution overnight was the optimal method to obtain single cell suspensions, which were minimally damaged and retained their clonal potential. From the cell suspensions that were prepared with this method, only the β1-integrin-dim and -bright cells had the ability to form substantial numbers of colonies. In the latter subpopulation more colonies were formed than in the first one. By means of the use of fluorescent DNA dyes, it was possible to visualise colonies at an early stadium. However, it turned out that keratinocytes could not stand up to the required concentrations of some of the dyes. By linking the area of the colony to the number of cells in that colony, it was possible to determine growth rates of the cells. In conclusion, after flow cytometric sorting with an optimised protocol, we were able to demonstrate that colonies were formed in the β1-integrin-dim and – bright subpopulation. These colonies could be visualised early with the use of fluorescent dyes. The use of image processing allowed early detection of colonies and determination of growth rates.

128

Fas/fas Ligand Pathway Involved in Arsenic-Induced Apoptosis in Cultured Keratinocytes

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Epidermiological studies demonstrated that long-term exposure to arsenic induces arsenical skin cancers, including Bowen's disease. Histologically, it shows proliferating and apoptotic characteristics at Bowen's disease lesion. To investigate how arsenic induces cellular apoptotic death, we exposed cultured human foreskin keratinocytes to different concentrations of sodium arsenite for 48 h, the expression of apoptotic proteins and receptors were detected by Western blotting and flow cytometry. Arsenic enhanced keratinocytes proliferation at low concentrations, however, apoptosis was induced at high concentrations (?5 μM). Arsenic enhanced p53 expression and arrested keratinocytes cell cycle at G0/G1 phase. The level of apoptotic protein Bax was increased in a Bcl-2 down regulated manner when keratinocytes were exposed to arsenic. The expression of several apoptosis related receptors were then detected, we found cell membrane Fas and Fas ligand (FasL) increased through arsenic treatment. Increased expressions of Fas related proteins such as FADD were observed. Caspase cascade was also activated as evidenced by increased caspase 3 and 8. Our results suggest that Fas/FasL pathway is involved in arsenic induced apoptosis in cultured keratinocytes.

129

Anti-Apoptotic Mechanisms in UV-Mediated Apoptosis: Cross-Class Inhibition of the Lysosomal Cystein Proteinase Cathepsin L by the Novel Ov-Serpin Hurpin (PI13)

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The novel serine proteinase inhibitor hurpin (proteinase inhibitor 13, PI13) has been linked to differentiation and apoptosis in human keratinocytes due to its repression after UV-irradiation and over expression in psoriatic skin lesions. Despite the fact that hurpin is a typical ovalbumin serpin with a hinge region that implicates an inhibitory capacity, a cognate target serine proteinase has yet not been identified. In order to determine the inhibitory capacity of hurpin we have expressed the recombinant histidine-tagged hurpin protein (rHP) in yeast and purified by metal chelat affinity- and subsequently ion exchange- chromatography. The yeast protein shows the serpin typcial transition from the stressed to the relaxed form as analyzed by transversal urea gradient gel (TUG) analysis. Until now, no inhibitory capacity of the rHP could be demonstrated for a variety of serine proteinases. In contrast the rHP was identified as a potent cross-class inhibitor of the archetypal lysosomal cysteine proteinase cathepsin L (CatL). rHP demonstrates a concentration dependent inhibition of CatL with a k_{ass} of $3.80 \times 10^5\ M^{-1}\,s^{-1}$. This rate constant implicates that hurpin is able to inhibit CatL at physiological concentrations. Also the leakage of cathepsins due to stress induced dysruption of lysosoms has been reported. Moreover lysosomal cathepsins are capable of activating executors of apotosis like caspase 3. The inhibition of CatL by hurpin is therefore further supporting the hypothesis of hurpin as an antiapoptotic protein and gives an example for possible physiological functions of serpins in the skin.

130

Development of a High-Throughput Model for Screening of Anti Psoriatic Drugs Using Expression of Green Fluorescent Protein Under The SKALP/Elafin Promoter

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Psoriasis is a common skin disease in which the cutaneous lesions are characterised by benign epidermal hyperplasia, abnormal differentiation, inflammation, and changes in the dermal vasculature. It is very likely that a combination of immunological predisposition (autoimmunity, superantigen stimulation) and keratinocyte abnormalities is key to the disease mechanism. It has been postulated that epidermal keratinocytes are actively involved in the pathogenesis of psoriasis. These cells form a signalling interface between the external and internal milieu by secreting cytokines and growth factors that act on inflammatory cells, and alter keratinocyte growth in a paracrine-autocrine manner. Keratinocytes of the psoriatic lesion are hyperproliferative and display an abnormal differentiation program characterised by the induction of genes that are not expressed by keratinocytes in normal skin such as the cytokeratins CK6, CK16, CK17 and the proteinase inhibitor SKALP otherwise known as elafin. Expression of these genes can be used as surrogate markers for psoriasis or other disorders with abnormalities in epidermal differentiation, in drug screening procedures of large compound libraries. A high-throughput screening model was developed for detection of pharmacologically active drugs in a 96-well culture model based on a permanent transfected keratinocyte cell line (HaCaT) that uses expression of the Enhanced Green Fluorescent Protein under control of the SKALP/elafin promoter. Induction of the SKALP promoter by TNFα, results in increased expression levels of the secreted SKALP-EGFP fusion protein. The model system will be further validated to define for which classes of drugs it can be used. Preliminary results indicate that these models can be used to monitor the effects of retinoids and MAPkinase inhibitors on cellular differentiation. This suggests that these models can be useful for screening drugs that act via the keratinocyte as the primary target cell, which could lead to improved pharmacotherapy of psoriasis and irritant contact dermatitis.

131

Irradiation with UV-Light Causes Downregulation of 12(S)-Hydroxyeicosatetra-Enoic Acid Receptors in Cultured Psoriatic Epidermal Cells

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On the cell surface, receptors could be affected during treatment with artificial UV sources or during skin irradiation for cosmetic reasons. Because 12-hydroxyeicosatetraenoic acid (12-HETE) is considered to be the main epidermal eicosanoid, and is assumed to have both pathophysiological effects in inflammatory skin diseases such as psoriasis and atopic eczema as well as a physiological role in cutaneous biology, we decided to show the UV-light effect on 12-HETE cell surface receptors. Therefore, the present work studied the effects of single and repeated irradiations with selected UV-B light of 313 nm from a Waldmann F 85/100 W – TL-01 bulb on the 12(S)-HETE receptors in psoriatic epidermal cells. UV-light *in vitro* (50–150 J per m^2) induced a down-regulation of 12(S)HETE receptors in a dose-dependent manner. The above described effect occurred after a latency period of 6 h and reached its maximum at 7.5 h. *In vitro*, a single UV irradiation (150 J per m^2) or repeated irradiation (50 J per m^2) developed a 55% receptor down-regulation (Bmax); however, the receptor affinity remained unchanged. The down-regulation of 12-HETE receptors on keratinocytes developed after the UV-B iradiation may contribute to the explanation of its effects in phototherapy or photoaging.

132

NF1 Tumor Suppressor Gene Expression is Markedly Aberrant in Psoriatic Skin *In Vivo* and During Cellular Differentiation *In Vitro*

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We have previously shown that psoriatic lesions display reduced tumor suppressor NF1 protein immunoreactivity in epidermis compared to the perilesional skin. In the present study, the expression of NF1 gene was studied *in vivo* and in psoriatic cell cultures using *in situ* (ISH) and northern hybridization, Western blot analysis and immunohisto-chemistry. The results showed that the level of NF1 protein was slightly reduced also in psoriatic cells *in vitro*. Moreover, psoriatic keratinocytes showed less intercellular variations than normal cells in immunolabeling with NF1 protein. ISH revealed that NF1 mRNA displayed reduced expression in lesional psoriatic skin compared to perilesional and normal skin. The subcellular distribution of NF1mRNA displayed also alterations in psoriatic keratinocytes during cellular differentiation *in vitro*. The results suggest that cultured nonlesional psoriatic keratinocytes were almost equally diseased as lesional cells. In contrast *in vivo*, marked differences were observed between lesional and perilesional skin in immunolabeling and *in situ* hybridization. NF1 protein is known to accelerate the inactivation of ras-GTP in various cell types. Thus our findings with aberrant distribution and expression of NF1 protein and mRNA may be causative to previously described increased activation of ras in psoriatic lesions. This finding is interesting because psoriasis is a proliferative disease with uncontrolled cell division, but lacking invasiveness and relative resistance to transformation.

133

Targetting NF1 mRNA During Cellular Differentiation

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We have previously demonstrated that NF1 tumor suppressor protein and cytokeratin 14 form a high–affinity interaction during the formation of cell-cell junctions in cultured keratinocytes, which were induced to differentiate by raising the extracellular calcium concentration. This prompted us to study the distribution of NF1 mRNA under the same conditions. The present study utilized cultures of human keratinocytes which can be used as a well documented cell differentiation model, and which have been successfully used for studies on expression and functions of the NF1 protein. Skin samples were obtained from operations carried out for cosmetic reasons from 10 healthy persons (aged 20–67 years). Keratinocytes (3rd to 8th passages) were used for experimentation. Prior experimentation, cells were seeded on slides for ISH experiments or on coverslips for protein detection and grown until about 40–60% confluence in serum-free low calcium keratinocyte growth medium. The medium was subsequently changed to defined KGM containing either low < 0.1 mM or high 1.8 mM calcium concentration. Cells were harvested at time points of 0, 0.5, 1, 2, 4, 8, 12, 16, 24 and 48 h and fixed. A NF1-specific 650 nt DIG-labeled cRNA probe was used for ISH. The cRNA/NF1 mRNA hybrids were detected by two alternative immunocytochemical methods. The findings of the present study highlight the fact that targeting of NF1 tumor suppressor mRNA takes place in contact forming and in migrating cells.

134

Analysis of Immunogold Particles Representative of Neurofibromatosis Type 1 (NF1) Protein in Human Epidermis

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Background We have previously shown that the NF1 Protein is associated with cytokeratin filaments of epidermal keratinocytes (Koivunen et al. 2000). In this study, we have investigated this association in more detail using pre-embedding immunoelectron microscopy and analyzing the distribution of immunogold particles in bundles of intermediate filaments.

Samples and methods 5 × 5 mm pieces of normal human skin were fixed in 3% paraformaldehyde for 2 h and incubated in sucrose. The tissue was frozen in liquid nitrogen and cut into 50–60 μm frozen sections. The primary antibody, NF1GRP(D), was incubated with skin sections for 2 h. The samples were then washed and incubated with goat anti rabbit IgG coupled to 10 nm gold particles. After the incubation and washes the sections were fixed with 5% glutaraldehyde and embedded in EPON 812. Numerical analysis of the distribution of immunogold particles were carried out using MCID-M4 (version 3.0, Rev 1.1, Imaging Research Inc., St. Catharine, Canada).

Findings The highest number of immunogold particles representative of NF1 protein in human skin was counted in the basal layer of the epidermis, while the spinous and granular layers were markedly less intensely labeled. Most of the immunogold was associated with bundles of cytokeratin within the cells. More specifically, most intense labeling for NF1 protein was located to the lateral aspects of cytokeratin bundles. Numerical analysis of the distance between two closest immunogold particles in keratinocyte was 48.1 ± 14.19 nm (SD). No immunogold particles were, detected in the extracellular matrix, attesting to the low level of unspecific background signal in the specimens analyzed. It should be noted that the signal for NF1 protein in the nuclei of keratinocytes hardly exceeded that of the background.

Conclusions The highest concentration of gold particles representative of NF1 protein were detected on the lateral aspects of cytokeratin bundles within the basal keratinocytes of human epidermis. Here, the distance between two closest immunogold particles was 48.1 nm. We speculate that this distance is somehow connected with the periodicity of cytokeratin bundles based on the 48 nm length of cytokeratin monomers.

135

Soybean Isoflavones and Endocrine Related Factors of Human Hair Follicle Cells

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Numerous studies have shown that non steroidal phytoestrogens (PHO) possess a broad spectrum of biological properties given them the potential to influence human physiology and hormone dependent-diseases such as inhibition of 5α-reductase activity in prostate cancer. The mechanisms by which these PHO exert their effect are tissue-specific and multifactorial. The impact of PHO on hair growth was less extensively investigated. These data led us to study firstly the effect of PHO on hair growth in organ culture and then to investigate the hypothesis that the hormonal responses of hair follicle cells *in vitro* are linked to the effects of PHO on 5α-reductase, aromatase and steroid receptors expression. The direct effect of PHO on hair growth was assessed on human isolated hair follicle. We evaluated by RT-PCR analysis the effect of a total plant extract: soya in comparison with two free forms (genistein, daidzein) and two conjugated forms (daidzin,puerarin) on the expression of type 1 5α-reductase gene expression. We also evaluated their effect on aromatase expression by cultured human hair follicle dermal papilla cells (DPC). In parallel, we evaluated by Western-blot and RT-PCR the effect of PHO on androgen and estrogen α and β receptors expression by DPC. Our results showed a significant inhibition of 5α-reductase gene expression in the presence of PHO (–21% inhibition for 1 μM genistein and –17% for 1 μM puerarin). Aromatase was seen to be markedly increased in the presence of genistein and daidzein (+76% stimulation for 1 μM genistein and +45% for 1 μM daidzein). Western-blot analysis showed a high stimulation of estrogen receptor β in the presence of PHO. The increase induced by soya extract at 10 μg per mL (+58.5%) is higher to that of 1 μM genistein (+43%) and 1 μM daidzein (+43% and +41% stimulation, respectively). PHO also promoted estrogen receptors α expression by DPC. However, the stimulation is less effective (+27% stimulation for 1 μM genistein, +9% for 1 μM daidzein and +15.5% for soya extract at 10 μg per mL). A decrease of androgens receptors protein expression is observed in the presence of PHO (–16% for 5 μM daidzein and –30% for 5 μM genistein). An improvement of hair follicle growth is observed in the presence of PHO (+30% hair growth with soya extract vs. controls). Our results suggest that PHO may act on the modulation of the main actors of testosterone metabolism in human hair follicle cells.

136

Altered Retinol Metabolism and Expression of Retinoid Receptors During Differentiation of Cultured Keratinocytes Results in Altered Retinoid Signaling

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Retinoids have profound effects on keratinocyte phenotype both *in vitro* and *in vivo*. These biological effects are primarily mediated by binding of the active metabolite all-*trans*-retinoic acid (RA) to nuclear retinoic acid receptors (RAR-α, -γ) which heterodimerize with 9-*cis* retinoic acid receptors (RXRs). These complexes might cause transcriptional changes directly or by interfering with other signaling pathways. Here, we examine retinoid signaling in cultured human keratinocytes during terminal differentiation induced by exposure to calcium, the phorbol ester PMA and interferon-γ (IFNγ). By using quantitative real-time PCR a time-dependent decrease in the mRNA expression of RAR-α/-γ and RXRα was found in cells exposed to both PMA and IFNγ for up to 48 h. In contrast, calcium-induced differentiation resulted in a 3-fold and 2-fold transcriptional up-regulation of RAR-γ and RXRα, respectively. Western blot analysis of nuclear extracts revealed concomitant changes in protein levels of the retinoid receptors. Both RAR-γ and RXR-α were induced by calcium but substantially reduced after 48 h exposure to PMA and IFNγ (to between 5 and 20% compared to control cells). Metabolism of [^{3}H]retinol to active RAR ligands as analysed by high-pressure liquid chromatography was increased by IFNγ and, more potently, PMA. Used as a marker for retinoid activity, the expression of the RA-inducible gene CRABPII, was rapidly and massively up-regulated at both mRNA and protein level by the phorbol ester but persistently down-regulated by IFNγ which might be due to titration of mutual coactivators. In all, our results show that retinoid signaling appear to be altered in differentiating keratinocytes but that the changes depend on how differentiation is accomplished.

137

Tight Junctions in Human Epidermis

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This study demonstrates the presence of tight junction components ZO-1 and occludin in adult and developing human epidermis. Indirect immunofluorescence labeling and immunoelectron microscopy with antibodies to ZO-1 and occludin localized tight junctions to a narrow zone of the granular cells of adult epidermis. Double immunolabeling for tight junction components and adherens junction or desmosome proteins suggested that occludin is more specific for tight junctions than ZO-1. In developing skin, tight junctions interconnected the peridermal cells, and after the fetal stratification localized to the granular cell layer. Immunolabeling of psoriasis, lichen planus and ichthyosis vulgaris, representing aberrant differentiation of the epidermis, showed that these conditions were associated with relocation of ZO-1 to the spinous cells. Cultures of differentiating epidermal keratinocytes revealed ZO-1 and occludin at the intercellular contacts. However, these tight junction proteins colocalized relatively late when the fusion zone had assumed a linear appearance. This suggests that the formation of adherens junctions and desmosomes precedes that of tight junctions. We speculate that the epidermal barrier, isolating the human body from external environment, is in part formed by tight junctions of stratum granulosum. Tight junctions may be important especially during fetal development and in pathological conditions affecting the epidermal lipid barrier.

138

Induction of Gap Junction Proteins in Epithelial Skin Tumours and Adjacent Epidermis

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Gap junctions are communicating channels that connect adjacent cells to allow communication by exchange of small molecules. They consist of two connexons (one of each cell), each formed by six transmembrane proteins called connexins. Fourteen different connexins (Cx) are known, seven of which are expressed in normal human skin. The expression of different connexins allowing specific molecules to pass through the channel thus controls the specificity of cell-cell-communication. In this work we concentrated on the expression of Cx30, Cx26 and Cx43 in epithelial skin tumours compared to normal interfollicular epidermis. Investigations were performed by immunofluorescence microscopy of frozen sections using previously described specific antibodies. As previously shown Cx30 and Cx26 are not present in normal interfollicular epidermis. In contrast Cx30 and at a lower degree also Cx26 are expressed in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Interestingly Cx30 is also found in normal interfollicular epidermis neighbouring SCCs. Similarily in certain cases there is an expression of Cx30 in the epidermis surrounding a malignant melanoma. But there is no expression of Cx30 in normal epidermis adjacent to BCCs. Cx43 is expressed in both epithelial tumours investigated, but at a lower degree than in normal epidermis. These results clearly show in epithelial tumours an induction of Cx30 and at a lower degree also of Cx26 and in contrast a down-regulation of Cx43. Therefore the induction Cx30 and Cx26 and the down-regulation of Cx43 seems to characterize activated and hyperproliferative tissue, which in consequence means, that Cx30 could be used as a marker for hyperproliferation. Ongoing investigations will elucidate the function of Cx30 in hyperproliferative tissues.

139

Desmosomes Exhibit Site-Specific Features in Human Palm Skin

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Mutations in genes encoding various desmosome (DM) constituents have recently been found to underlie a number of hereditary skin disorders characterized by abnormal thickening of palmoplantar skin. These sites may be preferentially involved because they are subjected to extensive physical stress. An alternative hypothesis is that there are instrinsic differences between DMs in normal palmoplantar skin and DMs in skin from other sites. We therefore compared a variety of morphological, immunohistochemical and biochemical properties of skin and cultured keratinocytes from normal human palm and breast samples. Using pan-antibodies directed against desmogleins and desmocollins, confocal microscopy showed that these DM cadherins exhibited a reciprocal graded distribution across the epidermis. Desmogleins were more abundantly expressed in the basal layer while the expression of desmocollins increased suprabasally. However, the expression profiles differed in detail between the two types of skin. In palm skin, from the mid-spinous layer upwards, staining for both DM cadherins as well as for desmoplakin was attenuated, suggesting a possible transitional change in DM organization. Staining for involucrin, a marker of terminal differentiation, started at the lower part of this zone, and was followed sequentially by the expression of transglutaminase, filaggrin, and finally, loricrin. By contrast, in breast skin, DM expression appeared more uniform across the epidermis and expression of the differentiation markers was confined to a much thinner zone nearer to the skin surface. A suspension-induced differentiation assay showed that involucrin expression occurred earlier in palm keratinocytes than in breast cells, and at 4–8 h, the number of involucrin-positive cells from the palm was almost twice the value obtained for the breast. Furthermore, Western blotting using cultured keratinocytes from both sites revealed increased expression of major DM constituent proteins in palm vs. breast cells. The differences in the expression of DM constituents between the two sites might account for the apparent 'dose' effects of certain mutant DM genes preferentially involving palmoplantar epidermis.

140

Expression and Localisation of Various Tight-Junction-Proteins in the Human Epidermis and Various Diseases

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Tight-Junctions (TJ) are cell-cell-junctions which are quite well characterized in simple epithelia and endothelia. There they form tight connections between the plasmamembranes of neighbouring cells to prevent the paracellular flux and to maintain cell polarity, therefore being a main contribution to the barrier function of these tissues. Interestingely, the composition of TJ varies in different tissues,seemingly reflecting their different barrier function. In human epidermis, existence, structure and composition of putative Tight-Junctions are still in discussion. In earlier reports we have shown the localisation of the Tight-Junction-proteins claudin-1, occludin and protein ZO-1 in human epidermis and cultured keratinocytes. Now we can show by RT-PCR that at least 6 more claudins are expressed in human skin/keratinocytes, even more stressing the striking high number of TJ-proteins and hinting for an important function of TJ in epidermis. To elucidate a possible barrier-function of TJ we investigated various diseases connected with an impaired barrier, e.g. eczema, psoriasis and wounding. We can show a change of TJ-protein expression. The data presented here indicate the existence of TJ-similar structures in human epidermis, especially in the stratum granulosum. Moreover, a contribution to the barrier function of the epidermis could be possible. Future experiments, especially electron-microscopy and dye transfer experiments shall elucidate this.

141

Desmocollin Isotypes in the Corneodesmosome; Processing During Stratum Corneum Maturation

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To investigate the expression of desmocollin (dsc) isotypes in corneodesmosomes and to identify the patterns of processing during desquamation we have used specific antisera raised against the N-terminal extracellular regions of the three human desmocollin isotypes. Tape strip depth analysis in association with immunoblotting and indirect immunofluorescence were used to identify and localise these proteins. All three isotypes were detected in human stratum corneum; dsc1 was the predominant form, although significant levels of dsc3 were detected while dsc2 was only a minor component. All three isotypes showed significant proteolytic processing and increased degradation towards the peripheral layers, with slightly different processing patterns for each variant. Indirect immunofluorescence revealed that dsc1 and dsc3 were localised on the corneocyte ridge regions, whereas dsc2 was more diffuse. Exhaustive extraction of the corneocytes to produce cornified envelopes demonstrated that desmocollins were cross-linked into this structure. Pertubation of desquamation with the soap-induced dryness model revealed, elevated levels of all three isotypes and their respective proteolytic fragments. Conclusion: (1) all three dsc isotypes were detected in human stratum corneum with dsc1 being the predominant variant; (2) all isotypes were extensively proteolytically processed with increased degradation towards the periphery; (3) significant levels of dsc were cross-linked into the cornified envelope; (4) dsc degradation was perturbed in soap-induced dryskin.

142

Calpain Isozymes 1 and 2 (CAPN1 and 2) and Calpastatin in Epithelial Human Skin Tumors

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Calpain, also named CAPN (for calcium activated neutral protease), represents an ubiquitious intracellular cytoplasmic nonlysosomal cysteine endopeptidase. Two major isozymes are known, named calpain 1 and 2, requiring micromolar and millimolar calcium concentrations for activation, respectively. Many substrates of the different calpain isoenzymes known today, such as the transcription factors c-Fos and c-Jun, the tumor suppressor protein p53, protein kinase C, pp60src, and the adhesion molecule integrin have been implicated in the pathogenesis of various human tumors including squamous (SCC) and basal (BCC) cell carcinomas of human skin, suggesting an important role of the calpain isoenzymes in malignant diseases. We have analyzed for the first time protein and mRNA expression of CAPN1 and CAPN2 in BCCs and SCCs of human skin. Interestingly, CAPN1 immunoreactivity (streptavidin-peroxidase technique) was markedly reduced in BCCs as compared to normal human skin (NS) or SCCs, while in contrast CAPN1 mRNA levels (real time PCR, LightCycler) were markedly elevated in BCCs (14,96 copies per unit) and SCCs (17,74 c per u) as compared to NS (o,135 c per u). No differences were found analyzing mRNA expression of CAPN2 (northern analysis) as well as immunoreactivity of CAPN2 and the endogenous CAPN-inhibitor calpastatin in NS, BCCs and SCCs. Our findings indicate that calpain isozymes may be involved in the tumorigenesis and growth regulation of BCCs and SCCs. Pharmacological modulation of calpain activity may be a target for the treatment of SCCs.

143

Analysis of the Vitamin D System in Basal and Squamous Cell Carcinomas of Human Skin

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Using real time PCR (LightCycler), we have analyzed basal cell carcinomas (BCC), squamous cell carcinomas (SCC) and normal human skin (NS) for mRNA expression of vitamin D receptor (VDR) and of the main enzymes involved in the synthesis and metabolism of calcitriol (vitamin D-25-hydroxy-lase [25-OHase], 25-hydroxyvitamin $D^{-1}\alpha$-hydroxylase [1α-OHase], 1,25-dihydroxyvitamin D-24-hydroxylase [24-OHase]). RNA for VDR, 25-OHase, 1α-OHase, and 24-OHase was detected in BCCs, SCCs and NS. Interestingly, RNA for VDR (BCCs: 35,2 copies per unit; SCCs: 44,7 c per u; NS: 0,07 c per u) and 25-OHase (BCCs: 0,94 c per u; SCCs: 34,63 c per u; NS: 0043 c per u) was up-regulated in BCCs and SCCs as compared to NS. Additionally, we performed conventional RT-PCR with total RNA from NS and BCCs to determine the expression of 1α-OHase. In both tissues, we report for the first time splice variants of 1α-OHase. Using a streptavidin-peroxidase technique and a panel of different monoclonal antibodies, protein expression of VDR and 24-OHase was analyzed immunohistochemically. Intensity of VDR and 24-OHase immunoreactivity was increased in SCCs and BCCs as compared to NS. However, staining did not correlate with histological type or grading of skin tumors. Comparing VDR-staining with staining for Ki-67, cytokeratin 10 and transglutaminase K, no correlation was found. Our findings indicate that (i) BCCs and SCCs may be considered as potential targets for prevention or therapy with new vitamin D analogs that exert little or no calcemic side-effects (ii) VDR expression is not exclusive-ly regulated by the proliferative activity or by the differentiation of these tumor cells, but by additional, unknown mechanisms (iii) synthesis and metabolism of vitamin D metabolites may regulate growth of BCCs and SCCs (iv) pharmacological modulation of vitamin D synthesis or metabolism may be a target for treatment of SCCs. The function of alternative transcripts of 1α-OHase that we here describe for the first time in BCCs and NS and its effect on activity level has to be investigated in future experiments.

144

Development of a Natural Whitening Agent from Macro Alguae Extract Undaria Pinnifitida

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The micro alguae Undaria pinnifitida (UP) are widely consume in Asian countries. Besides its nutritional qualities owing the presence of amino acids, polyols, vitamins and oligoelements, it has attracted special interest for medicines such as anti viral, anti cancer or antibiotics… Using it as the starting material, three aqueous fractions were obtained after ultrafiltration, and tested for their activity on mushroom tyrosinase. The maximum enzyme inhibition (43%) was registered with the UP3 fraction. We then examined depigmenting efficiency by inhibition of melanogenesis of human melamona cells (M4Be). We measured a decrease of melanin synthesis by 35%. Complementary analysis of other macra alguae extracts demonstrated its specificity of action. In addition, the UP3 fraction was evaluated on human adult melanocytes (a decrease in melanin content of 55% was obtained at 1 mg per mL) to confirm the compound's suitability for use in cosmetic skin-whitening products.

145

Human Melanocortin 1 Receptor (MC1R) Variants are Restricted in their Ability to Stimulate cAMP in B16-G4F Cells

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Case control and kindred studies have demonstrated an association between variants of the human melanocortin 1 receptor *(MC1R)* gene and red hair and fair skin. Previous studies investigating signalling of human MC1R receptor variants have employed transient transfections of cell lines which are not related to the melanocyte lineage (e.g. COS and HEK293). Studies on the function of MC1R variants using primary melanocytes are also limited by a lack of control over background genetic variations. The mouse B16-G4F melanoma cell line does not constitutively express murine Mc1r. We therefore generated stable B16-G4F transfectants with wild type human MC1R and separately with Arg151Cys and Arg160Trp variants using electroporation and subsequent selection with G418. Stably transfected cell lines expressing wild type MC1R ($n = 3$) were able to significantly increase cAMP production when stimulated by α melanocyte stimulating hormone at concentrations of 10^{-6}–10^{-9} Molar. By contrast, the transfected Arg151Cys ($n = 3$) and Arg160Trp ($n = 4$) B16-G4F cell lines were unable to stimulate cAMP production to the same extent, despite a similar cAMP response to 3-Isobutyl-1-methylxanthine (IBMX) as that seen in the wild type MC1R transfected cell lines. The results show that MC1R variant receptors are compromised in their ability to signal via cAMP when expressed within cells of the melanocyte lineage.

146

Patterns of Single Nucleotide Polymorphism's (SNP's) in the Melanocortin 1 Receptor (MC1R) Gene

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The melanocortin 1 receptor (MC1R) plays a key role in human pigmentation. Individuals homozygous or compound heterozygous for the Arg142His, Arg151Cys, Arg160Trp or Asp294His variants of *MC1R* have red hair and a lighter skin colour, a phenotype which is thought to be subject to selective pressure. Population studies of a number of SNP's within *MC1R* have revealed strong functional constraint of the gene in Africa and relaxation of this constraint in populations outside of Africa, for example in Europe and Asia. To further analyse selection of *MC1R*, we have examined 2Kb of DNA flanking the gene and have studied the patterns of these polymorphisms in a number of Caucasian ($n = 31$), African ($n = 27$) and Asian ($n = 9$) alleles. 12 SNP's were identified, 8 within 1100 bp of DNA 5′ of *MC1R* and 4 within 800 bp of DNA 3′ of *MC1R*. No SNP's were identified in the second exon of *MC1R* (*MC1RB*). The African population was the most polymorphic, in contrast to the results of SNP's within the MC1R gene. A number of haplotypes spanning *MC1R* (951 bp) and the flanking SNP's (2Kb) were inferred statistically and these were used to propose a gene tree of probable mutation events. Several MC1R variants (Arg142His, Arg151Cys and Asp294His) were exclusively associated with one haplotype, although it was not possible to attribute this to selection.

147

β-Carotene Uptake and Bioconversion to Retinol is Different in Human Melanocytes and Keratinocytes

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Epidemiological studies have associated a high dietary intake of β-carotene with reduced cancer risk. In addition to being a vitamin A precursor, β-carotene has antioxidant properties and increases the immune response. These functions are important in the defence against ultraviolet induced skin cancer. Human skin contains high levels of both β-carotene and vitamin A. It is not known if β-carotene can be converted to vitamin A in the skin and thereby affect cellular differentiation and growth in this tissue. We used primary cultures of human keratinocytes and melanocytes to study the uptake of authentic or ^{14}C-labelled β-carotene and its possible bioconversion to vitamin A. The levels of β-carotene and vitamin A were analyzed with HPLC technique. The uptake of β-carotene was much higher in melanocytes than in keratinocytes corresponding to a 5-fold difference in the intracellular fraction after 2 days of incubation. An increased level of cellular vitamin A was noted after 1 day of β-carotene incubation. The conversion of [^{14}C]β-carotene to [^{14}C]vitamin A peaked at 24 h of incubation in both keratinocytes and melanocytes. In conclusion, melanocytes are especially prone to store β-carotene which is converted to vitamin A in human melanocytes and keratinocytes. Local storage together with conversion of β-carotene to vitamin A in the skin, might be two powerful processes protecting the outer shield of the body towards UV induced skin cancer.

148

The Role of Human Myosin Va Isoforms in Human Epidermal Melanocytes

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The actin based motor Myosin Va is a homodimer consisting of two 190 kDa myosin heavy chains, each with an N-terminal head domain provided with an actin-and ATP- binding site and a relatively long neck domain. The two heavy chains subsequently dimerize into a central coiled coil stalk followed by the globular tail, with a presumed role in cargo binding. The role of this motor protein in melanosome transport was partially elucidated when studying pigmentary dilution in mouse dilute mutants and patients with the autosomal recessive Griscelli syndrome. Previously we discovered that the human Myosin Va produces different transcripts in a cell type specific manner, with splicing occurring in the C-terminal portion of the gene. The biological role of the isoforms found in human epidermal melanocytes is still unknown. Therefore, we constructed GFP-fusion proteins (pEGFP-C1 vector) comprising the human Myosin Va C-terminus alternative transcripts. Confocal microscopy on human epidermal melanocytes transiently transfected with GFP fused to six different exon combinations of the domain where alternative splicing occurs, reveals a diffuse pattern throughout the cytoplasm with a dense perinuclear localization, excluding the nucleus. Five constructs consisting of GFP, five different exon combinations and the entire downstream C-terminal tail show a distinct specific pattern of localization, again excluding the nucleus. The localization pattern of these five constructs can be defined in three categories. ACE and ABCE accumulate into a distinct perinucleair dot. ABCDEF and ACDEF are concentrated in the cortex of the plasma membrane and in the periphery and the tips of the dendrites. ABCEF is present in distinct linear organized dots throughout the cytoplasm and the dendrites. To obtain detailed information about the exact subcellular (co)-localization of the isoforms, immuno-electron microscopy with an anti – GFP antibody on transfected melanocytes is currently performed. Our observations indicate that the alternative splice domain has a profound influence on the specificity in localization of the tail region. The difference in localization probably involves a difference in function of the transcripts.

149

Establishment and Characterization of an Immortalized Human Epidermal Melanocyte Cell Line

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We report the isolation and partial characterization of a spontaneously immortalized human epidermal melanocyte cell line (SPAG). Human epidermal melanocytes were isolated and cultured from normal skin of a healthy Caucasian female undergoing plastic surgery. The evolution of indefinitely growing melanocytes appeared spontaneously. These melanocytes have been passaged over 75 times to date and show no signs of senescence after 1½ years of *in vitro* culturing. These cells are dendritic, tripolar and bipolar, showing typical melanocyte morphology. SPAG cells have a diploid kariotype with an isolated defect of the 15th chromosome. This kariotype appears to be stable with repeated passages. We studied the expression of several cell cycle and melanocyte/melanoma associated genes in SPAG cells. We detected overexpression of Cyclin D1 protein, a specific marker for G1 cell cycle phase. Tyrosinase expression was verified with nested RT-PCR in several passages. Messenger RNA expression of MIA (melanoma inhibiting activity, a newly recognized melanoma specific marker) was not detectable in any passage. This immortalized human epidermal melanocyte cell line (SPAG) could be an important new tool for the study of diverse aspects of melanocyte biology.

150

Gene Expression Profiling with cDNA Microarray Technology of Ultraviolet-B Irradiated Normal Human Melanocytes. Relation to Melanocarcinogenesis

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Target genes involved in ultraviolet B (UVB) stress response of cutaneous melanocytes, some of them potentially involved in melanoma development, were identified by cDNA microarray (MA) technology applied to one-third of the human genome. Cultured normal human melanocytes (NHM) were subjected to an accute UVB irradiation (100 mJ per cm^2). Four hours later, mRNA were extracted from UVB- and sham-irradiated (control) NHM. Respective mRNA populations were reverse transcribed for hybridization to 9000 cDNA of human genes on MA. Analyse of hybridization signals allowed to select 198 genes with a significant differential expression (DE) = 1.9. 81 were over-expressed, while 117 were under-expressed. Among them, 159 corresponded to DNA known sequences, the encoded proteins being mostly involved in DNA- or RNA-binding/synthesis/modification and ribosomal proteins. The others were transcription factors, receptors, channel-related proteins, and tumor suppressors. All of these protein function-families housed some UVB sensitive genes that have been already involved in melanoma. 39 modulated DNA sequences were Expressed Sequence Tags (EST), waiting for gene characterization and protein function. Target known genes were located on all chromosomes but showed a preferential distribution. Some chromosomes bearing UVB sensitive genes were known to harbor melanoma loci. Expression studies in melanoma cells and tumors of some selected EST, as well as comparison of our data with those from other MA studies in melanoma samples, strongly suggest that several modulated genes play a collective role in melanocarcinogenesis. Our study shed light on melanoma candidate genes on a large genome scale and encourage us to characterize some of these EST, two useful steps for further molecular epidemiology investigations and identification of "gene cluster predictors" in melanoma.

151

Expression of Retinoblastoma Gene in Melanoma Cell Line

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The high incidence of melanoma (4–14%) in retinoblastoma patients suggest that an oncosuppressor gene, the retinoblastoma gene could play a role in the progression of melanoma. Therefore we have studied the expression of retinoblastoma gene in three melanoma cell lines. Cell culture of 3 melanoma cell lines was performed in Mac Coy 5 A medium, 5% of CO_2. ARN extraction and amplification was done by RT-PCR. Western blot was performed with an antihuman pRb monoclonal antibody (G3-245). The level of protein was quantified by spectrophotometry. We confirm the expression of mRNA of retinoblastoma gene and of the protein Rb in 3 new melanoma cell lines. Despite a different tumorigenicity the level of expression of protein was identical in all cell lines and positive control MOLT-4. Thus our 3 cell lines express Rb protein at a pre and post transcriptional level. Quantitative RT PCR could assess wether there is or not a significant difference between the cell lines and positive control. We cannot exclude the possibility in some cell lines of a dysfunction of Rb protein secondary to mutations of other molecules or genes (CDKN2/p16…).

152

Screening of Melanoma Patients for the Presence of Cross-Reactive Autoantibodies Against Autologous Tumour and Retinal Tissue

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Melanoma-associated retinopathy (MAR) is a paraneoplastic syndrome in metastatic cutaneous melanoma. The pathomechanism is understood to result from antibody production against melanoma-associated antigens which cross-react with epitopes of retinal cells. We recently analyzed 20 melanoma patients in different stages of disease (stage I-IV, according to the AJCC tumour classification) for the presence of cross-reactive antibodies against autologous tumour and retinal tissue. Autologous patient's serum and a polyklonal rabbit-antiretina serum were used to determine the autoantibody activity on histological sections of primary melanoma, melanoma metastases, and retinal tissue, respectively. Using a fluorescein isothiocyanate- (FITC) conjugated secondary antibody, a rapid evaluation of the immunologic activity of the serum sample was permitted. Interestingly, most of the serum samples contained high titers of antibodies that cross-reacted with retinal tissue as well as with autologous tumor. Our results showed that the percentage of reactive serum samples was increased in advanced stages of disease and that the presence of autoantibodies often was an initial symtom leading to the clinical detection of metastases. The production of MAR-antibodies seems to be insufficient to protect against the dissemination of melanoma cells. Furthermore the presence of MAR-antibodies is associated with a worse prognosis. These data strongly indicate that a particular melanoma-associated antigen is able to induce the production of autoantibodies that may cross-react with retinal tissue and may cause the development of MAR in certain cases.

153

Tissue Counter Analysis of Tissue Components in Skin Biopsies – Evaluation Using Cart (Classification and Regression Trees)

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In tissue counter analysis, complex histologic sections are overlayed with regularly distributed measuring masks of equal size and shape, and the digital contents of each mask (or tissue element) are evaluated by grey level, colour and texture parameters. In this study we assessed the feasability of tissue counter analysis and classification and regression trees for the quantitative evaluation of skin biopsies. From randomly selected skin biospies ($n = 100$) a learning set of tissue elements was created, differentiating between cellular elements, collageneous elements of the reticular dermis, fatty elements and other tissue components. Classification and regression trees based on the learning set were used to automatically classify tissue elements in samples of normal skin ($n = 25$), benign common nevi ($n = 50$), malignant melanoma ($n = 50$), molluscum contagiosum ($n = 25$), and scleroderma ($n = 25$). The procedure yielded reproducible assessments of the relative amounts of tissue components in various diagnostic groups (t-test: $p < 0.05$). Furthermore, a reliable diagnostic separation between molluscum contagiosum on one hand, and normal human skin on the other, and between malignant melanoma on one hand, and benign common nevi on the other, was possible with 100% sensitivity. Tissue counter analysis combined with classification and regression trees are a suitable approach to the fully automated analysis of histologic sections of skin biopsies.

154

How Should Dermatological Digital Images be Prepared for Presentation, Publication and Teledermatology

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There is an increasing opportunity to handle digital images for dermatology in presentation, publication and even teledermatology. It is essential to standardize a quality of digital images including a focus, a level and a contrast. First to keep high quality in these images are most important, however, unnecessarily huge images often make your personal computer freeze. Second, digital images are always unfocused when you scan them by image scanners. We should therefore optimize the size and focus of these images when we use them for different purposes. As a standard monitor has pixels of 1024×768, this or a little less size is thought to be a suitable size for a slide for a presentation. In teledermatopathology and teledermoscopy, diagnoses are usually made on the monitor and the same size is enough and necessary. On the other hand, the images for publication need more pixels according to the journal to which you submit a paper. When the publisher requests the print size of 5×3.5 inches, you need pixels of 2000×1500 at 400 dpi color printer of high quality. This size is being achieved by a recent digital camera of 3.5 M pixels. The most prominent feature of the digital image is feasibility of image correction. Concerning the image processing, we recommend to use a photo retouching software, like PhotoShop ®. When a pathological image in an electronic format is obtained by scanning the image on a slide using a slide scanner or taken by a digital CCD camera directly connected to a microscope, it is inevitable that the digitized image is unfocused and low in contrast. The main process of improving the image is to use a level correction, a tone curve control and an unsharp masking. These procedures should properly be used in order to recover the original image and should not be used to produce unnecessary enhancements. In the present study, we have compared examples of printed images of different quality and the images before and after an image processing. Although there was no apparent difference between images printed either at 200 or 400 dpi regarding clinical images, figures containing lines seemed to need the higher quality. Images sent for teledermatopathology have greatly been improved after an appropriate image processing.

155

Pattern Recognition Analysis of Human Cutaneous Mast Cell Populations by Topographical Total Body Surface Mapping

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Mast cells (MC), important sentinel cells in immediate type hypersensitivity reactions, are abundant in tissues that interface the external environment, including the skin. However, little is known about the influence of age, sex, or skin region on size or spatial distribution of MC populations in normal human skin. Here, we have performed extensive histomorphometric entire body surface mapping of healthy human skin, analysing MC numbers and distributions in 15 distinct anatomical skin sites. MC per microscopic field (MF) were quantified in ≥ 5 different skin layers (subepidermal to subcutaneous) by light microscopy ($\times 400$) in toluidine blue-stained 3 µm sections of tissue samples obtained from ≥ 10 individuals (total n: f = 87, m = 63, ages 10–86) for the following anatomical sites: trunk (T) = abdomen, thorax, lower or upper back; proximal extremities (PE) = lower or upper arm, lower or upper leg; distal extremities (DE) = foot, hand; and face (F) = chin, nose, cheek, forehead, back of head. In all skin sites, regardless of age or sex, MC numbers in distal skin layers were higher than in proximal skin layers (maximum: subepidermal = 9.3 ± 0.4 MC/MF, minimum: subcutaneous = 1 ± 0.1 MC/MF). No differences in total skin MC numbers and skin layer specific MC numbers were found comparing skin obtained from male or female and from young or old individuals, respectively. Interestingly, total skin MC numbers were highest in distal skin sites (DE and F; maximum: nose = 9.4 ± 0.9 MC/MF) and lowest in proximal skin sites (T; minimum: abdomen 2.9 ± 0.3 MC/MF), while PE exhibited intermediate MC numbers (upper arm: 3.7 ± 0.2 MC/MF, lower arm 4.9 ± 0.3 MC/MF). These data show that cutaneous MC in healthy human dermis localize preferentially to distal skin layers and sites. One possible explanation for these unexpected findings is that skin site-specific factors such as exposure to UV irradiation or minimal trauma are involved in the regulation of skin MC numbers.

156

CD44 and Hyaluronate in the Differential Diagnosis of Dermatofibroma and Dermatofibrosarcoma Protuberans

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The histological distinction between dermatofibroma (DF) and dermatofibrosarcoma protuberans (DFSP) may be extremely difficult. CD34 and Factor XIIIa have been used to differentiate DF from DFSP. However, there is an overlap and lack of specificity of their expression. CD44 is a widely distributed integral membrane glycoprotein which is expressed as a multitude of isoforms generated by alternative splicing of at least 10 different variant exons and post-translational modifications. CD44 is currently thought to be the principal cell surface receptor for hyaluronate (HA), the major component of the extracellular matrix. In this study we aimed to assess the expression of standard CD44 (CD44s) and its isoforms (CDv3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v7v8 and CD44v10), and HA in DF and DFSP. Immunohistochemical staining was performed on the biopsy specimens of 15 cases of DF and 4 cases of DFSP, using antibodies which recognize the CD44s and different CD44 isoforms, and hyaluronate binding protein (HABP). Tumor cells displayed a strong CD44s immunoreactivity in all cases of DF whereas a faint HA positivity was observed in the tumor stroma. DF cells were negative for CD44v3, CD44v4, CD44v6, CD44v7 and CD44v7v8 but they showed a strong reactivity for CD44v5 and CD44v10. In contrast, CD44s expression was significantly reduced or absent in all DFSP lesions and the tumor stroma displayed a strong staining for HA. Our results indicate that CD44 and HA can be used as additional diagnostic markers to distinguish DF from DFSP.

157

Differentiation of Lichen Sclerosus from Morphea: Three Dimensional Reconstruction of Basement Membrane Zone and Vascular Network in Laser Scanning Confocal Microscopy

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Morphea and lichen sclerosus (LS) are separate entities in spite of not infrequently overlapping clinical and histological patterns. Ultrastructural studies on LS showed changes in upper dermis and alterations of basement membrane zone (BMZ) on the level of lamina densa. The present study was undertaken to compare alterations of various regions of the BMZ and vascular network in morphea and LS using laser scanning confocal microscopy. The study included three cases of morphea, three cases of LS and three cases overlapping clinical and histological criteria of both diseases. Biopsies from patients' skin and a control biopsy from normal human skin were cut into 20 μm thick slides and labeled with antibodies against β-4 intergin (lamina lucida marker), collagen IV and N-terminal end of collagen VII (lamina densa markers) and C-terminal end of collagen VII (sublamina densa marker) using routine immunofluorescence. Three-dimensional reconstruction of various regions of BMZ showed decreased number and size of the dermal papillae in both LS and morphea as compared to normal skin. In morphea, flattening of BMZ on the level of lamina lucida, lamina densa and sublamina densa were observed but continuous character of BMZ was preserved, whereas numerous invaginations and holes were present in LS BMZ. Vascular skin network system visualized by labeling with anticollagen IV antibody revealed increased angiogenesis in cases of early morphea, as compared to LS and normal skin. Patients overlapping clinical and histological patterns of morphea and LS reflected alterations of BMZ and vessels characteristic for both diseases. Three-dimensional reconstruction of BMZ and skin vascular network revealed different alterations in morphea and LS and its may serve as a valuable investigative tool for differentiation of both entities, especially in the onset of morbid process where is a diagnostic doubt.

158

Clone Specific *In Situ* Hybridization for Preparation of Malignant Cells in Cutaneous T cell Lymphoma

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Clonally dominating T cells represent the malignant cells in cutaneous T cell lymphoma (CTCL). However, identification of these cells in skin and blood samples from CTCL patients is hampered by the lack of a specific tumour marker. Here we report on a clone specific *in situ* hybridisation technique specifically labelling the malignant cells in paraffin embedded tissue and in cytospins using antisense RNA gene probes directed against the individual, tumour specific T cell receptor (TCR) β gene rearrangement. This approach should greatly facilitate the separation and subsequent investigation of the tumour cells. According to the unique sequence of the clonal TCR β rearrangement, a clone specific TCR β DIG-labelled RNA probe was designed for the Jurkat cell line and 2 CTCL patients as well and the cells were stained following hybridisation with BCIP/NBT. The specificity of the method was demonstrated by controls using corresponding sense RNA probes, cross hybridisations on sections of other CTCL patients, having the same V β family rearrangened, and, by dilution experiments of Jurkat cells in other clonal T cell lines. Using our method the tumour cells can be observed directly by fluorescence microscopy or can be isolated by flow cytometry. This in turn facilitates further investigation of their biology giving more insight into etiology and pathogenesis of CTCL. In addition, the technique is well-suited for the estimation of tumor cell frequencies in blood or tissues.

159

Distribution of Cytochrome P450 (CYP) 2e1 in the Skin: A Novel Marker for Keratiocyte Differntiation?

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CYP2E1 is known to be induced in human liver after alcohol intake and recently, expression in the human keratinocytes was reported. The purpose of the present study is to investigate the expression of CYP2E1 in the skin from normal subjects and some dermatological disorders including psoriasis and skin tumors. To detect the CYP2E1 protein expression, immunohistochemical method using antiserum for CYP2E1 is employed. In the normal epidermis, the protein distributes mainly upper 2/3 part and weak positive staining was observed in the basal layer. In the dermis, vascular endothelium and eccrine sweat gland except myoepithelium were well stained. Skin specimens from involved psoriatic skin showed a similar pattern. Positive staining was also observed in the keratinizing part of squamous cell carcinoma. Basal cell epitheliomata were negatively stained. Contrary to the neighboring normal eccrine gland, eccrine spiradenoma shows negative result. In Paget's disease, nested tumor cells were negative while surrounding keratinocytes were well stained. Together with these results, the enzyme protein expression seems to be dependent on the cellular differentiation and could be used as discriminator for determination of surgical margin.

160

Balneotherapy Modify Iron and Ascorbic Acid Concentrations in Atopic Dermatitis Dermis

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Atopic dermatitis (AD) is a chronic inflammatory skin disease induced by reactive oxygen species (ROS). ROS formation is catalysed by iron and they are scavenged by ascorbic acid (AA). The aim of this work was to determine iron and AA concentrations in atopic dermatitis patient dermis before and after a balneotherapy and to compare them with iron and AA levels in healthy subject dermis. Five AD patients and eight healthy subjects were enrolled in this work. Iron and AA were collected from human dermis by microdialysis and assessed, respectively, by atomic absorption spectrometry and gas chromatography-mass spectrometry. Iron concentration in AD dermis was more than twice the iron level in healthy subjects dermis (44.3 ± 4.6 μg per liter, 21.4 ± 2.3 μg per liter, respectively; $p \leq 0.05$). AA level was lower in AD dermis (46.7 ± 0.6 μg per mL) than in control dermis (237.4 ± 8.7 μg per mL; $p \leq 0.05$). Iron concentrations decreased and AA level increased after balneotherapy treatment (35.0 ± 4.7 μg per liter; 67.0 ± 5.1 μg per liter, respectively). However, iron and AA concentrations were quite significant different than iron and AA levels before the treatment ($p = 0.06$). These results suggest that iron and AA levels could be an indicator of inflammatory tissues and could be implicated in dermatological diseases such as DA.

161

p53 Gene Analysis in Single Cells From Human Skin

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The advanced laser capture microdissection (LCM) techniques have made it possible to exploit targeted cells from histologically stained sections without contamination from neighbouring cells. When combined with optimized techniques for gene amplification and sequencing, detailed questions relating morphology to genetic background can be addressed. In an effort to obtain optimized conditions for p53 gene analysis in single cell, we investigated various strategies for sample preparation and gene amplification. As a result we can amplify six individual exons of the p53 gene (exons 4–9) in 70% of isolated single cells. In 50% of these cells both alleles are amplified. p53 mutations were characterized in chronically sun-exposed skin from healthy individuals. Biopsies were taken from skin which had been subjected to daily summer sun as well as adjacent skin which had been totally protected from the sun by blue denim fabric (SPF 1700). 172 single-cell samples were retrieved from 4 biopsies. A total of 14 different mutations were identified in 26/99 keratinocytes from which the p53 gene could be amplified. Immunohistochemical staining revealed a small cluster of p53 immunoreactive keratinocytes suggestive of an epidermal p53 clone, with a diameter of 10–15 basal cells. Two missense mutations (codon 241 and 281) were found in all layers of epidermis within the p53 clone. In addition rare keratinocytes within the p53 clone contained additional mutations. In normal skin we also found scattered single cells with typical UV-induced mutations. The presented data show that p53 mutations are common in normal skin and that a clone of keratinocytes with a mutated p53 gene prevailed despite two months of total protection from ultraviolet light.

162

Fluorescence Imaging of Epithelialization from Skin Explant Cultures on Acellular Dermis

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Reconstructed skin models *in vitro* are valuable tools in morphological studies of epidermis, but are less suitable for quantitative analysis of the reepithelialization process. We describe an experimental model which permits sequential measurement of the epidermal outgrowth rate whilst maintaining the skin tissue in culture and allowing histological or immunohistochemical sampling. A 2-mm skin-punch explant was used as keratinocyte source and attached to the centre of a circular 8-mm piece of deepidermized dermis, DED. The combined tissue sample was placed on top of a modified CellStrainer filter and cultured at the air–liquid interface in serum-containing keratinocyte medium. By this means a multilayered epithelium expanded from the grafted skin tissue, resurfacing the upper aspect of the DED within a 10–14-day period. Without terminating tissue culture, the area of epidermal outgrowth was visualized repeatedly by vital staining using fluoroprobes at subtoxic concentrations. The usefulness of the method was tested by studying the effect of hrEGF in the culture medium. It was found that supplementation with hrEGF at 10 ng per mL not only induced increased immunostaining for the proliferation marker Ki-67 and acanthosis of the newly formed epidermis, but also enhanced the epithelialization rate as determined by fluorescence microscopy and digital image analysis. When treated with hrEGF, the time to attain 75% reepithelialization of the DED was reduced by approximatively 30%. Thus, the combined effects of rEGF on the resurfacing rate, proliferation index, and histology of neo-epidermis were demonstrated using one and the same experimental setting. As compared to existing epidermal culture models this technique permits concurrent analysis of skin epithelialization dynamics and morphology. The advantages of this simple and miniaturized skin culture model might be useful in studies of skin epithelialization.

163

Lipoma-Like-Liposarcoma of the Skin in the Course of Xeroderma Pigmentosum

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Male patient aged 16 years with clinical diagnosis of Xeroderma pigmentosum.Skin lesions were lentigo, freckles, discolouriastion and hyperpigmentation appeared after the first year of life due to exposure to sunlight. The number of skin changes was increasing with the patient age. At 6 years he was referred to the Institute of Oncology, where he was treated for numerous foci of carcinoma basocellulare. At 8 years he was admitted for the first time to the Department of Dermatology PAM. Next the patient was treated many times for baso- and squamous carcinomas. The last hospitalisation of the patient was in September 2000. The nodule (1 cm in diameter) was noted on the skin of the patients nose with the crust on the surface. The examination revealed changes on the entire skin involving freckles, lentigo and in uncovered sites numerous foci of excessive keratosis, hyperpigmentation, discolourisation dryness and thickening of the skin as well as conjunctivitis with photophobia. Lymph nodes were not enlarged. It was phisical retardation wheares psychological developement was normal. Genetic examination showed diminution of DNA synthesis in lymphocytes. Histopathological examination no. 68620 of the skin tumor on the tip of the nose revealed lipoma like liposarcoma.

164

A Clinicopathologic Study of Basosquamous Carcinoma

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1108 patients with skin cancer underwent surgery between 1993 and 1999, at the Department of Dermatology and Venereology Pomeranian Medical Academy in Szczecin. Histopathologically, there were 92 (8.3%) cases of basosquamous carcinomas, 800 (72.2%) basal carcinomas and 206 (19.5%) squamous carcinomas. Basosquamous carcinomas were located mainly (77 cases – 84%) on the skin of the face (usually cheek, nose and angle of the mouth), the remaining 15 cases (16%) involved the extremities (forearm, thigh and dorsum of the hand). Histopathologic examination in 92 cases of basosquamous carcinomas revealed the presence in the dermis histologic features of basal carcinoma and squamous carcinoma (cellular atypy, cancer pearls) together. The electron microscopic study of 6 basosquamous carcinomas revealed the lack of continuity in the basement membrane and keratin pearls within the cytoplasm of keratinocytes. All patients underwent safety margin surgery, the curative effect of which was permanent.

165

Long-Term Nicotinamide (NA)-Treated HaCaT Keratinocytes Display Enhanced Differentiation and Decreased Growth Capacity

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NA, a derivative of B3 vitamin and precursor of NAD, is known as inhibitor of poly ADP ribose polymerase (PARP) and weak free radical scavenger. In the present study we examined the effect of continuous cultivation (6 months) of HaCat keratinocytes in presence of 10 mM or 20 mM NA on cell differentiation, proliferation and death. Indirect immunofluorescent method was used to determine keratin 10 and involucrin expression; cornified envelope formation by light microscopy was also studied. Cell proliferation was estimated with MTT assay, growth curve was examinated by light microscopy and cell cycle analysis by FACS. Cell death was investigated by fluorescence microscopy (apoptosis and necrosis) and flow cytometry (DNA fragmentation). Expression and cleavage of PARP was estimated by Western Blot analysis. In earlier studies we demonstrated antiproliferative and differentiation-promoting effects of short-term NA treatment (72 h) on HaCat keratinocytes. In the present study we found that long-term cultivation (6 month) with 10 mM and 20 mM NA resulted in dose-dependent increase of keratin 10 and involucrin expression and cornified envelope formation in HaCat cells. Growth-curve analysis and MTT assay demonstrated 2- to 4-fold decrease in cell proliferation of long-term HaCat cultures treated with 10 mM and 20 mM NA compared with control cells. NA caused accumulation of HaCat cells in S and G2/M phase and increased level of apoptosis ($4.5 \pm 0.38\%$ in control compared to$10.2 \pm 0.11\%$ and $14.9 \pm 0.93\%$ in cells treated with 10 mM and 20 mM NA, respectively). Long-term cultivation with NA did not induce activation of necrotic death of HaCat cells. NA-treated cells showed no alteration in level of PARP expression compared with untreated cells. In addition, we did not find cleavage of PARP following NA treatment. Our results indicate that long-term treatment with NA (6 months) has strong potential to inhibit the proliferation and to stimulate the terminal differentiation in HaCat keratinocytes.

166

***In Situ* Detection of a Novel Costimulatory Molecule, Dectin-1, on Dendritic Cells in Psoriasis**

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We have currently identified by using subtractive cDNA cloning technologies a dendritic cell (DC) specific molecule with costimulatory functions, Dectin-1 and –2, both of which belong to C-type lectin receptor family. Besides their specific expression on DC and the capacity to selectively mediate UV-induced tolerance in experimental animal models, it is entirely unknown how Dectin-1 (Dec1) is regulated in diseased conditions in human. Recently, accumulating evidence suggest that pathogenesis of psoriasis may be attributed to T cell-mediated immunity which consequently leads to proliferation of epidermal keratinocytes. Based on the fact that DC are the major player to induce immune responses in skin and that Dec1 is specifically expressed on DC, we were interested to study the expression of Dec1 on psoriasis. Therefore, specimens derived from skin of patients suffering from psoriasis ($n = 15$), from atopic dermatitis ($n = 8$) or of normal healthy individuals were analyzed for expression of Dec1 by double-immunostaining techniques using an antibody directed against human Dec1 and an antibody against CD1a, respectively. Consequently, it was found that the numbers of $Dec1^+CD1^+DC$ are significantly increased in both dermis and epidermis of psoriatic skin, while only the less number of the double positive DC were observed in healthy skin or in atopic skin. Interestingly, while fluorescence intensity of CD1a remained rather unchanged between the samples tested, that of Dec1 was markedly highered on DC in psoriatic skin as compared to those of atopic or healthy skin, indicating the induction of the Dec1 expression on DC in psoriasis. Together, these data imply the possible role of Dec1 in pathogenesis of psoriasis.

167

Detection of T-Cell Receptor γ Sequences in Patients with Primary Cutaneous CD30+ Large T-Cell Lymphomas by Micromanipulation and Single-Cell PCR

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The CD30 antigen was first identified in 1982 by Stein et al. as a cell surface marker expressed by Hodgkin and Reed-Sternberg (H/RS) cells in Hodgkin's disease (HD). This antigen was soon recognized to play a role in various physiological as well as pathological processes in the organism. The expression of CD30 is mainly restricted to cells of the immune system, especially lymphocytes. As a member of the TNFR superfamily the molecule is assumed to be involved in the process of lymphoid development and differentiation. Several studies indicate CD30 to be a mediator of cell growth as well as apoptosis depending on different immunological stimuli. However, the exact mechanisms of CD30 signalling are still unknown. Concerning the diagnosis and prognosis of different lymphoproliferative disorders such as HD and anaplastic large cell lymphoma (ALCL) the antigen is of particular interest. In the case of primary cutaneous lymphomas the detection of the antigen led to the classification of a new type of lymphoma characterized by the predominance of CD30+ tumor cells and a favourable prognosis. The CD30 positive cutaneous large T-cell lymphoma (CD30+ CTCL) is defined to consist of large lymphoid tumor cells which in at least 75% are positive for the CD30 antigen. A pleasant 5 years survival rate of 90% and the tendency of this lymphoma to spontaneous regression (in up to 25% of the cases) is thought to be connected with CD30 signalling. Although the large CD30+ lymphoid cells are accepted to represent the tumor cell population, this assumption has not been proven on a molecular-biological level yet. We believe the protein to be a very important participant in the pathogenesis of CD30+ CTCLs. The aim of this work is to answer the question whether the CD30+ cells in primary cutaneous CD30+ large T-cell lymphomas belong to the tumor cell population (and if they exclusively do so) or not. Therefore we isolated CD30+ cells of three patients with CD30+ CTCLs. A total of 212 CD30+ single cells was acquired by hydraulic micromanipulation. 185 of the cells were picked from the epidermis of tumor lesions and 27 from a cytospin slide of peripheral blood lymphocytes. In order to identify clonal cell populations, which are thought to represent the tumor cells, we employed single cell PCR of the T-cell receptor (TCR) γ gene followed by direct sequencing. In 119 of the 212 separated cells we succeeded in analysing a rearrangement for the TCR γ. In all three cases a clonal population with a biallelic rearrangement of the TCR γ was detected. Beside these clonal populations which comprise the majority of the isolated cells, all samples showed the existence of CD30+ cells with polyclonal rearrangements. Additionally one patient showed a second small cell clone. From the previous work we could prove for the first time in CD30+ CTCLs that the tumor cell population is to be found within the group of CD30+ large lymphoid cells. According to our results we conclude that the CD30+ large T-lymphocytes do not represent a uniform tumor cell population, although the morphological and phenotypical occurrence is equal between clonal and other T-lymphocytes. With regard to the varying but not insignificant amount of polyclonal CD30+ T-cells within the tumor lesions their possible role in the pathogenesis of CD30+ CTCL should be considered.

168

Differential Expression of the CD10 Antigen (Neutral Endopeptidase) in Primary and Metastatic Malignant Melanomas of the Skin

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The CD10 antigen (neutral endopeptidase, enkephalinase, NEP-24.11:EC 3.4.24.11) is a 100-kDa cell surface metalloendopeptidase which inactivates by hydrolysis a various proinflammatory peptides. It was initially considered as specific of lymphoblasts of acute lymphoblastic leukemia (CALLA), but is now known to be expressed by a variety of normal and neoplastic nonlymphoid tissues. In a previous study we observed that the CD10 antigen was expressed by a subset of cutaneous malignant melanomas (MM). In this study we investigated the expression of the CD10 antigen in a wider variety of MM of the skin in order to get further insight into the significance of this molecule as a progression marker, and to evaluate a potential usefulness of the CD10 antigen in dermatopathology. An immunoperoxidase technique was applied on formalin-fixed, paraffin-embedded tissue specimens of 72 MM (including 28 primary, 26 metastatic to the skin and 18 lymph-node metastases). The CD10 antigen was expressed by 18/26 (69%) and 11/18 (61%) of MM metastatic to the skin or the lymph nodes, respectively; by contrast, only 6/28 primary MM (21%) expressed appreciable CD10 reactivity, which was usually lower (in terms of percentage of immunoreactive cells) than that found in metastatic MM. The sensibility, specificity, positive and negative predictive value of CD10 positivity for metastatic MM was evaluated to 0.66, 0.79, 0.83 and 0.6, respectively. These results suggest that the CD10 antigen is up-regulated during the process of metastasis in MM. Whereas the explanation for this up-regulation is as yet unclear, the expression of CD10 appears as an additional immunohistological feature for the differential diagnosis between primary and metastatic MM.

169

Tumor Thickness, Ki-67-Antigen and Polo-Like Kinase (PLK) Expression in the Assessment of Proliferative Activity and Prognosis of Thin Malignant Melanomas (=0.75 mm)

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The maximum thickness of malignant melanomas is currently the most important prognostic factor. In recent years the tumor thickness of excised melanomas has decreased. In Central Europe, nearly 50% of tumors examined nowadays are less than 0.75 mm thick. However, these thin melanomas, which should have an excellent prognosis, can metastasize and can be lethal. Therefore, better prognostic tools are required to identify thin melanomas with high metastatic potential. Ki-67 is a nuclear antigen expressed in the G1-, S-, G2-, and M-phase of the cell cycle. Polo-like kinase (PLK), named after the Drosophila gene product polo, codes for a serine/threonine kinase and regulates multiple steps of the cell cycle, e.g. the spindle formation, cyclin-dependent kinase activation and M-phase exit. 22 thin metastasized melanomas and 14 thin melanomas from patients without disseminated disease were analyzed with immunohistochemical techniques using monoclonal antibodies directed against the proliferation associated antigens PLK and Ki-67. Two independent operators evaluated stained sections. The PLK and Ki-67 indices were calculated as the number of positive cells per 1000 totally counted cells (multiplied by 100%). The maximum tumor thickness was assessed by Breslow's method. Differences of tumor thickness, Ki-67 and PLK indices between melanomas with and without metastases were tested statistically using the Mann–Whitney *U*-test. Neither tumor thickness ($p = 0.885450$) nor Ki-67-antigen expression ($p = 0.150473$) provided a significant prognostic result with respect to metastases. In contrast, malignant melanomas with metastases expressed PLK at markedly elevated levels compared to melanomas without metastases ($p = 0.000053$). Our results demonstrate that the assessment of Polo-like kinase (PLK) expression in immunohistochemical sections reflects the metastatic potential of thin malignant melanomas (= 0.75 mm).

170

Elastin Peptides Upregulate Prommp-2 Activation and Melanoma Cells Migration through Three-Dimensional Type I Collagen Matrix

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Elastin, as a main constituent of elastin fibers, plays a prominent role in skin biology. Its enzymic degradation by gelatinases (MMP-2, MMP-9) leads to the liberation of elastin peptides (E.P.) which exhibit several biological functions. Particularly, interaction between E.P. and a truncated β galactosidase elastin/laminin receptor present at the plasma membrane of various cell types was shown to trigger MMP-expression (Brassart et al. *J Biol Chem* 2000). We here evidenced that elastin peptides (50–200 μg per mL) from organo-alkaline or elastase hydrolyses of insoluble elastin could stimulate MMP-2, but not MMP-1, expression from highly tumorigenic melanoma cells, in a dose-dependant manner. When those cells were grown within type I collagen gels, in presence of EDPs, MMP-2 activation was strikingly exacerbated, an effect probably resulting from elastin-mediated MT1-MMP increased expression (Brassart et al. *Clin Exp Metastasis* 1998). Parallely, invasion of type I collagen matrix by melanoma cells was significantly increased. This preliminary investigation, in keeping with our previous data (Capon et al. *Clin Exp Metastasis* 1999), indicate that the main fibrillar matrix macromolecules of human dermis i.e collagen and elastin actively contribute to tissue invasion, through modulation of MMPs expression and activation, by melanoma cells.

171

Smad3 is Involved in Regulating the Transforming Growth Factor-β Elicited Expression of Collagenase-3 (MMP-13) in Gingival Fibroblasts

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Collagenase-3 (MMP-13) is a collagenolytic matrix metalloproteinase with a wide substrate specificity, and it is expressed by fibroblasts in gingival wounds. TGF-β is a potent inducer of MMP-13 gene expression in gingival fibroblasts, and this requires the activation of p38 mitogen-activated protein kinase pathway. In this study, the role of Smads, the signaling mediators of TGF-β, in regulating the expression of MMP-13 in gingival fibroblasts has been studied. We have constructed recombinant HA(haemaglutinin)-tagged Smad2, –3 and –4 -adenoviruses, and shown by Western blotting that they express the corresponding transgenes in infected gingival fibroblasts. Immunostaining analyses show that the HA-tagged Smads are translocated into the nucleus after 2 h TGF-β1 treatment of infected fibroblasts, indicating that the adenoviruses express functional Smads. Western and Northern blot analyses show that overexpression of Smad3 enhances the TGF-β1 elicited expression of MMP-13, which is further induced when Smad4 is coexpressed with Smad3. In comparison, the TGF-β1 induced levels of TIMP-1 (tissue inhibitor of MMPs) and type I collagen remain unchanged in response to Smad3 and –4. In addition, transient transfections with human MMP-13 promoter and Smad expression vectors indicate that the activity of MMP-13 promoter is enhanced by Smad3 and –4. These results provide evidence that Smad3 is involved in regulating the expression of MMP-13 in gingival fibroblasts.

172

Induction of Keratinocyte MMP-9 Expression by Boron and Manganese

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Synthesis of new tissue and maturation of wound by remodelling of the extracellular matrix (ECM) are two phases involving matrix metalloproteinases (MMPs). Leif R. Lund et al. have reported the presence of MMP-2, –3 and –9 mRNAs in wound healing but in different patterns. In addition, somme clinical studies on trace elements lead to demonstrate their beneficial effect on wound healing. Thus, boron compounds would induce wound healing by acting on ECM and on the synthesis and secretion of TNF-α. An induction of integrin expression by proliferating and differentiated keratinocytes after manganese treatment was also described by authors. In the present study, we have investigated the effect of boron and manganese on MMP-2, –3 and –9 keratinocyte expression. Normal human keratinocytes were cultured in monolayers and incubated with boron (0.5-1-5-6.46–10 μg per mL) or manganese (0.1–0.2–0.3–0.6–1.5 μg per mL) for 6 h. Cytoplasmic expression of MMPs was analysed in triplicate by immunohistochemistry. This study revealed an increase in MMP-9 cytoplasmic expression by keratinocytes incubated with 10 μg per mL of Boron and more than 0.2 μg per mL of Manganese after 6 h of incubation. No modulation of MMP-2 and –3 was noted. During wound healing, MMP-9 mRNAs are just located at the front of the migrating epidermal layer until 7 days after wounding. Application of these trace-elements on scars could improve cell migration by their action on MMP-9 expression in keratinocytes of the migrating front and could explain the clinical results obtained with these trace elements.

173

Serum Levels of Tissue Inhibitor of Metalloproteinases 2 in Serum of Systemic Sclerosis Patients – Preliminary Results

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Systemic sclerosis (SSc) is characterised by increased deposition of collagen and other components of extracellular matrix (ECM) in skin and internal organs. It is not elucidated whether increased production of collagen or its degradation disturbance is responsible for this process. Metalloproteinases (MMPs) are regarded as important enzymes in both proteolysis processes of ECM components and angiogenesis. MMPs activity is regulated by their tissue inhibitors (TIMPs). The latter are postulated to be involved in neoplastic processes, rheumatoid arthritis and systemic sclerosis. Tissue inhibitor of metalloproteinase 2 (TIMP-2) can block many enzymes such as interstitial collagenase, gelatinase and stromelysin, which are in turn involved in ECM metabolism. The aim of our study was to measure TIMP-2 levels in serum of SSc patients and correlate them with extensiveness of skin and lung changes evaluated by spirometric method. The study was performed in the group of 18 SSc patients (8 with limited – lSSc and 10 with diffuse type – dSSc; 12 female and 6 male, aged 16–70 years). The control group comprised 10 healthy people.TIMP-2 in serum was measured by ELISA method according to the manufacturer (Amersham, Buckinghamshire, England). It was demonstrated that mean TIMP-2 level in serum of SSc patients was slightly increased in comparison to the control group (80.07 ng per mL vs. 77.63 ng per mL, respectively). There was no statistically significant difference between lSSc patients and dSSc ones (81.42 ng per mL vs. 78.99 ng per mL, respectively, at $p > 0.05$). There was no correlation observed between duration of Raynaud's phenomenon and the degree of skin fibrosis and serum TIMP-2 levels. Increased TIMP-2 serum levels were observed only in patients who had ventilation problems of restrictive nature demonstrated on spirometric examination. The obtained results seems to support the probable role of TIMP-2 in lung fibrosis development in systemic sclerosis patients.

174

Human Serum Components Increase Laminin 5 Synthesis in Human Keratinocytes

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Laminin 5 is a major component of the anchoring filaments that connect epithelial hemidesmosomes with the basement membrane. In wound healing, laminin 5 is the first basement membrane component deposited under migrating keratinocytes, and it is known to enhance keratinocyte migration. Cultured human keratinocytes were concentration-dependently stimulated to produce laminin 5 by treatment with human acute wound fluid, as well as human serum, to almost the same extent. Human serum was fractionated by Sephacryl S-300 column chromatography and the effect of each fraction on laminin 5 synthesis by human keratinocytes was examined. Activities were mostly separated into three groups, high-molecular-weight, IgG, and albumin fractions. High-molecular fractions, in which fibronectin is a major component, enhanced keratinocyte adhesion and increased laminin 5 synthesis in keratinocytes. The active component may be fibronectin, since keratinocytes plated on fibronectin synthesized more laminin 5. IgG fractions also increased laminin 5 synthesis, although release of laminin 5 into the medium was lower than with the other fractions. The active component in this fraction has not yet been identified. Albumin fractions increased laminin 5 production and its release into the medium. The active components might be albumin-binding lipids such as lysophosphatidic acid, lysolecithin and sphingosin-1-phosphate, which increased laminin 5 synthesis in a concentration-dependent manner, and cytokines released from platelets, such as TGFα and TGFβ1, which also stimulated laminin 5 synthesis. Moreover, laminin 5 production in keratinocytes was also augmented by an inflammatory cytokine, TNFα, which may be present in acute wounds. These results suggest that wound fluid may facilitate wound repair by stimulating laminin 5 synthesis in keratinocytes.

175

The Collagen Cross-Link Hisitdino-Hydroxylysinomerodesmosine is an Artifact

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An appropriate cross-linking of collagen molecules is an essential prerequisite for proper biomechanical function of connective tissues. The cross-link histidino-hydroxylysinomerodesmosine (HHMD) has been described as a tetrafunctional cross-link derived from lysine-aldehyde pathway and was suggested as a major cross-link in skin. An increase in the concentration of HHMD has been shown for sclerotic skin in localized scleroderma and keloids, while a decrease has been reported for lipodermatosclerosis. However, there is no convincing evidence that HHMD is formed under *in situ* conditions. In this study, we analysed the cross-link pattern of skin and other HHMD-containing tissues by different analytical procedures. Human tissues (normal skin, keloids, palmar aponeurosis) underwent various treatments for cross-link analysis. Untreated tissues were reduced by borohydride and then digested by collagenase or firstly digested by coallgenase and then reduced by borohydride. After hydrolisation (6 N HCL for 24 h at 108°C) cross-links were analysed with an amino acid analyser. Cross-link analysis yielded in all samples similar levels of reducible and nonreducible cross-links except HHMD, which was solely found in the samples which were reduced prior to collagenase digestion. Our study demonstrates, that HHMD is not present in tissues *in situ.* HHMD is an artifact which is generated during reduction of tissues by borohydride. The analysis of HHMD may be indicative for the content of the intramolecular aldol condensation product (ACP), which plays no role in the mechanical properties of the fibre.

176

Role of Integrins in Regulation of Dermal Scar Formation in Humans

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Cutaneous wound healing is a complex process that involves migration and proliferation of various cell types including keratinocytes and fibroblasts. Various cytokines and molecules of provisional extracellular matrix (ECM) within the dermis regulate functions of fibroblasts. The purpose of the study was to evaluate the role of integrins and several extracellular matrix components during dermal scar re-organization. Serial biopsies of human wounds healed by primary intention were obtained from 26 patients who underwent repeated surgery (in a period of 7–35 days) due to the histological diagnosis of melanoma *in situ.* The biopsies were analyzed by means of immunohistochemistry with the use of anti $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, αv, $\beta 1$ and $\beta 3$ molecules. The most pronounced changes were observed between days 7 and 35 in the expression of $\alpha 2$, αv and $\beta 1$ integrins subunits, as well as in the vitronectin expression. The $\alpha 2$ integrin was up-regulated on dermal fibroblasts between days 7 and 27 after injury. After day 27 the $\alpha 2$ subunit expression gradually decreased. The αv and $\beta 1$ integrins expression were up-regulated between days 7 and 35 on fibroblasts and endothelium. The vitronectin expression was increased from day 7 onwards and colocalized to the area of the wounded dermis similarly to the αv subunit expression pattern. These data suggest that human dermal scar fibroblasts use mainly $\alpha 2$, αv and $\beta 1$ integrin subunits during re-organization of ECM. Changes in the integrin expression may play an important role in the control of deposition of ECM components associated with scar remodeling.

177

Positive Correlation Between Catalase Activity and Collagen Synthesis in Suction Blister Fluid

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Chronically sun exposed and severely photodamaged skin shows a reduction in collagen synthesis, while acute UV-irradiation leads to an increase in metalloproteases. These changes could be mediated by reactive oxygen species (ROS) and/or DNA damage. In this study we evaluated the endogenous levels of various antioxidant defence systems together with markers of collagen metabolism. Thirty samples of suction blister fluid were collected on the dorsal forearms of human volunteers. The suction blister fluid was analysed for its level of reduced glutathione (GSH) and catalase activity. Collagenase activity (MMP-2 and MMP-9) was quantified and collagen synthesis levels were detected as the aminoterminal propeptides of type I and type III procollagen (PINP and PIIINP). A positive correlation was found between the catalase activity and GSH levels in suction blister fluid ($p = 0.01$). We observed a positive correlation between the catalase activity and the levels of PINP and PIIINP (p-values are 0.03 and 0.01, respectively). There was no correlation between the catalase activity (or GSH levels) and the collagen breakdown rate when measured as MMP-2 and MMP-9. Evaluation of these data suggests that a low antioxidant status measured as a low catalase activity and GSH content in suction blister fluid coincides with a low collagen (I and III) synthesis rate. These findings support the *in vivo* damaging role of ROS, and hydrogen peroxide in particular, which may contribute to the reduction in collagen synthesis.

178

Evidence for Activation of Classical Pathway of Complement in the Noninvolved Skin of Patients with Systemic Lupus Erythematosus

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The presence of immunoglobulins at the dermo-epidermal junction (DEJ) in patients with lupus erythematosus (LE) is known as the lupus band. In a previous study we have demonstrated that the lupus band is relatively restricted to IgG3 subclass which has the longest hinge region, making it more readily available for C1q. Binding C1q may either activate the classical complement pathway with the formation of membrane attack complex (MAC) or activation restricted to the early complement components C1 and C4. Inactivation of C4 produce C4d which remains covalently attached to the target as a stable marker for complement activation via the classical pathway. Previous studies have demonstrated the prescence of MAC in the DEJ in lesional skin but not in normal appearing skin of patients with discoid LE or systemic LE. The aim of this study was to investigate the presence of C4d in nonlesional skin of patients with systemic LE to detect prior reactions in which MAC are not demonstrated. This may provide insight into the role of complement in cutaneous LE. Biopsies from noninvolved skin from 20 systemic LE patients were investigated by indirect immunofluorescence. Skin sections were incubated with C4d Moab then with FITC-conjugated rabbit antimouse antibody to detect the deposition of C4d. Normal skin obtained from healthy individuals were used as a control. In 18 out of the 20 biopsies C4d was detected. C4d appeared as an intense bright fluorescent band at the DEJ and in the dermal blood vessels. C4d was not detected in the normal control skin. The presence of C4d within normal appearing skin of patients with systemic LE implies activation of complement via the classical pathway. Activation of complement up to and including the cleavage of C4 may have a protective role and may partly explain why MAC is not formed in the nonlesional skin of LE patients. Currently we are investigating the role of complement control proteins in cutaneous LE.

179

Antinuclear Autoantibodies in Blood for Transfusions – a Possible Risk Source For Recipients?

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The example of neonatal lupus erythematosus teaches us that transfer of autoantibodies may cause irreversible effects in the recipient's body. This study was aimed at assessing the prevalence of autoantibodies in blood for transfusion. Blood samples of 50 donors from a donation centre in Lublin (eastern Poland) were tested. The donors were all males aged 21–55 (median 35) years, none was suspected of having autoimmune disease. Enzyme immunoassay was used with the Varelisa ReCombi ANA Profile EIA kit (Pharmacia, and Upjohn) with the following antigens: dsDNA, RNP, Sm, SS-A/Ro, SS-B/La, Scl-70, CENP, Jo-1. Results: one or more antinuclear autoantibodies were detected in 6 of 50 blood samples (12%); dsDNA-specific autoantibodies were found in 1, RNP – in 1, SS-A/Ro – in 4, and Scl-70 – in 1 sample. No Sm, SS-B/La, CENP, and Jo-1-specific autoantibodies were detected. We conclude that a closer analysis of possible impact of autoantibodies in transfusion blood on recipients' health should be undertaken, in order to decide if screening blood donors for autoantibodies would be reasonable.

180

Increased Hydrogen Peroxide Exhalation by Systemic Sclerosis Patients

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Respiratory system disturbances are one of the major death causes in systemic sclerosis (SSc) patients. Pathomechanism of lung changes is still not fully elucidated. It is assumed that gatherings of activated fagocytes, which are regarded as a source of reactive free radicals (RFR) are observed in places of vasculitic and fibrotic changes. Quantity of expired hydrogen peroxide (H_2O_2) could be regarded as RTR disturbance exponent. The aim of our study was to correlate quantity of the expired H_2O_2 with the degree of lung function damage in systemic sclerosis patients. Twenty-three SSc patients were included in the study (14 with limited form – lSSc and 9 with diffuse – dSSc). Results of spirometric tests (FVC, FEV_1 and FEV_1/FVC in percentage of the required values) and H_2O_2 levels in condensate of the expired air obtained in SSc patients were correlated with the values of respective parameters obtained in the control group. Condensate of the expired air was collected between 8.00 and 12.00 a.m. in all the examined patients. Spectrofluorometric method was employed to evaluate H_2O_2 level in the condensate. Spirometric examination was performed on the same day. Spirometric results demonstrated that ventilation restriction of the restriction type was observed in 8 SSc patients, of obstruction type in 7 patients and of combined type of 3 SSc patients. There were no changes in 5 SSc patients and in all healthy controls. In SSc patients FVC%, FEV_1% and FEV_1/FVC% values were as follows 75.87%, 61.86% and 78.74%, respectively, and in healthy controls 104.25%, 97.75% and 91.11%, respectively (differences between the groups were statistically significant at $p < 0.05$ for FVC% and FEV_1%). Mean H_2O_2 levels in the expired air condensate were 810.94 nM in all SSc patients and 343.77 nM in the control group ($p < 0.05$). The obtained results seem to support the role of RFR in SSc pathomechanism. Increased levels of H_2O_2 in the expired air condensate demonstrate oxidative stress events in the respiratory tract and correlate with the degree of lung function impairment. Measurements of H_2O_2 could be regarded as a noninvasive, adjuvant examination in lung function evaluation in SSc.

181

Regional Variations in Anisotropy of Skin Viscoelasticity in Japanese Women

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The measurement of running time of acoustical shear wave propagation is excellent for obtaining skin mechanical properties in a specific direction *in vivo*. Recently a device based on this principle has been applied for examining multidirectional viscoelasticity in Caucasian forearm skin. In this study we studied skin viscoelasticity and anisotropy in forearm and facial skin in Japanese females using this newly developed device. A total of 105 healthy Japanese females (10 s–70 s: 15 subjects to each decade) were examined in this study. Measurements were taken for the inner surface of the left forearm in three locations (upper, middle and lower parts), the middle area of the left cheek and the center of the forehead while extending the arm parallel to the body in the supine position. The running time of acoustical shear wave propagation between unit distances were evaluated using Reviscometer (C + K Co., Ltd). Measurements were taken at angles of 0/180°, 45/225°, 90/270°, and 135/315° oriented against the direction of body axis by rotating the sensor probe with steps of 45° counterclockwise. An overall average of the running time recorded in four directions in the forearm decreased from the upper to the lower part, but were longer than those in the cheek and forehead, indicating reduced stiffness in forearm skin. In a mutual comparison of the mean running time in all directions, although the forehead showed no differences between each direction, the cheek showed a maximum value at 45/225° and a minimum value at 135/315°. Furthermore in the upper, middle and lower parts of forearm, maximum values were observed at 90/270°, 135/315° and 0/180° and the minimum values were observed at 45/225°, 45/225° and 90/270°, respectively. The findings in this study show the regional variations in skin viscoelasticity and anisotropy in these areas, and suggest that this measurement is useful for evaluating the characteristic of skin mechanical properties in each area of the body.

182

Age-Related Changes of Skin Viscoelasticity and Anisotropy in Japanese Women

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Measurement method based on the running time of acoustical shear wave propagation in a multi direction has been applied for examining age-related changes in mechanical properties of Caucasian forearm skin. In this study forearm and facial skin in Japanese females was measured with the newly developed device using this method, changes in skin viscoelasticity and anisotropy with age were investigated. A total of 105 healthy Japanese females (10 s–70 s: 15 subjects to each decade) were examined in this study. Measurements were taken for the inner surface of the left forearm in three locations (upper, middle and lower parts), the middle area of the left cheek and the center of the forehead while extending the arm parallel to the body in the supine position. The running time of acoustical shear wave propagation between unit distances were evaluated using Reviscometer (C+K Co., Ltd). Measurements were taken at angles of 0/180°, 45/225°, 90/270°, and 135/315° oriented against the direction of body axis by rotating the sensor probe with steps of 45° counterclockwise. Statistically significant age-related changes of running time in a defined direction were noted in areas except the upper part of the forearm. In the forehead an increasing tendency was seen at 0/180° and 45/225°, and a decrease at 45/225° together with an increase at 135/315° with age was observed in the middle area of the left cheek. Furthermore in the forearm, age-dependent decrease was shown in all directions except 135/315° in the lower part and 0/180° in the middle part. When the coefficient of variation of values recorded in four directions was calculated and plotted as a function of age, only the middle area of the cheek showed a significant decrease age-dependently, indicating reduction in the anisotropy of skin viscoelasticity with age. These results reveal that age-related changes of viscoelasticity vary with direction and the area of skin, and suggest that this measurement is useful for evaluating the variation in skin anisotropy with age.

183

Specific Furans from Avocado: A New Tool in Dermatology

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Based on our know-how in the fields of lipidic extracts, we have discovered original and specific compounds from avocado, namely Avocadofuran. The aim of this work is to present this new and original molecule, and especially its biologicals activities on both the dermis and the epidermis. Avocadofuran (INCI name: heptadecadienyl furan) is obtained from virgin avocado oil. The drying of the entire fruit is an important step in the process which allows the selective formation of Avocadofuran. This molecule is then concentrated in a two steps molecular distillation process. Molecular distillation is a physical process of fractioned evaporation carried out under high vacuum, and used for heat-sensitive high molecular weight and low volatility products. This long chain furanic lipid does not have any equivalent in the field of natural compounds. Studies were first performed on normal human skin fibroblasts. Avocadofuran (20 μg per mL) was added directly in cell culture medium. After 24 h incubation, TGFβ-1 production was measured in cell culture medium using a specific ELISA kit assay. In our experimental conditions, Avocadofuran was able to significantly induce TGFβ-1 synthesis compared to non treated cells. This increase in TGFβ-1 has been related to an increase in collagen synthesis as demonstrated by the collagenase assay. This result was confirmed using antibodies against TGFβ. Indeed, when cells were incubated with these antibodies, the effect of Avocadofuran on collagen secretion was almost completely abolished. In a second set of experiments, we have checked the effect of Avocadofuran on epidermal lipids synthesis using human skin explants. We have demonstrated that this molecule was able to stimulate specifically the synthesis of cholesterol and ceramides 1 and 2, without any modification of the total lipid content of the epidermis. Taken together, these results demonstrate that Avocadofuran is able to stimulate skin metabolism, both at the dermal and epidermal level of extra cellular matrix synthesis, especially for the development of a new generation of topical products intended for the prevention and/or the treatment of skin aging.

184

HCR, a Candidate Gene for Psoriasis, is Expressed Differently in Psoriatic Epidermis Compared with Normal Skin

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Psoriasis is associated strongly with the HLA-Cw6 allele. We have shown that one of the genes near HLA-Cw6, named HCR for α helical coiled coil rod protein, is highly polymorphic and associates strongly with psoriasis in Finnish patients making it a good candidate gene for the psoriasis susceptibility locus PSORS1. To further investigate the role of HCR in psoriasis, we performed immunohistochemistry with rabbit polyclonal antibody on 31 psoriatic lesions, 13 samples of nonlesional skin and 5 biopsies of normal skin. 16 of the psoriatic lesions were untreated, 10 had been treated locally and 5 with peroral medication or UVB. The cytoplasm of basal keratinocytes in normal skin stained positively with also some nuclear staining suprabasally. Nonlesional skin did not differ from normal skin, showing cytoplasmic staining basally in 11/13 samples and suprabasal nuclei were positive in 7/13 samples. In lesional skin, expression was up-regulated within nuclei and cytoplasm not only basally but also suprabasally above dermal papillae with attenuated expression in other parts of the epidermis. Positive HCR staining was detected in all psoriatic untreated lesions and was not appreciably changed in the lesions treated locally with corticosteroids or calcipotriol. The hyperproliferation marker Ki67 showed inverse patterns of staining compared to HCR. In contrast to psoriasis, HCR distribution in lichen planus, eczema, and pityriasis rubra pilaris was similar to nonlesional or normal skin.

185

Identification of Differences in Gene Expression Between Nonlesional Psoriatic and Healthy Epidermis

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Mechanical injures and T-cell derived cytokines are well-known inducers of psoriasis on the noninvolved skin of psoriatic patients but not on healthy individuals (Bata-Csörgõ, 1995). In order to find out the cause of this phenomena we investigated the differences between nonlesional psoriatic and healthy epidermis at the transcription level. Differential display (DD) analysis was performed comparing the mRNA expression pattern of the nonlesional epidermis of 2 psoriatic patients and the epidermis of 2 healthy individuals. 25 differentially expressed fragments have been cloned, analysed by restriction endonucleases and by reverse Southern blot then were sequenced. Sequence homology searches revealed that the genes for a *ras* oncogen, RAB10 and fibronectin are up-regulated in the nonlesional psoriatic skin. The oncofoetal form of fibronectin had been already reported to have an elevated mRNA level in the nonlesional psoriatic epidermis (Ting, 2000), proving that the used DD method is a powerful tool in our experimental setup. The rest of the cloned DD fragments showed sequence homologies to cDNAs with yet noncharacterized functions as well as to genomic sequences. The role of these genes in the proliferation and differentiation processes of human epidermal keratinocytes are studied in a synchronized HaCaT cell model by RT-PCR. Both RAB 10 and fibronectin showed a proliferation-related expression pattern. Additionally, we demonstrated the expression of the newly identified genes in the immortalized HaCaT keratinocytes: the expression of several genes was characteristic to discrete proliferation or differentiation states of the culture while some genes were expressed constitutively during the development of cell culture. Using the DD technique we were able to detect already known and yet unknown up-regulated mRNAs in the noninvolved psoriatic skin. The characterization of the genes for these newly identified transcripts and further analysis of their expression may bring us closer to the understanding of the genetic background of the hyperproliferative skin disease, psoriasis.

186

Endogenous Retroviral Proteins are Differentially Expressed in Normal Human Skin and Psoriatic Skin

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Human endogenous retroviruses are classified into 22 families and constitute 2% of the human genome, but their roles have not been characterized yet. However, increasing evidences suggest that they could participate during tumor progression and in the pathogenesis of autoimmune diseases, including multiple sclerosis or polyarthritis rheumatoid. We have investigated whether these sequences were expressed in normal and pathological skin samples including psoriasis by using two sera directed against the transmembrane protein of the moloney mouse tumor virus that contains highly conserved regions within the retroviral family. In protein extracts from normal human skin, we observed by western-blot two major proteins at 63 and 60 kDa and minor bands at 85, 57 and 29 kDa. These proteins were mainly detected in the insoluble pool suggesting that they were localized in the membrane or in the cytoskeleton. The different size observed could correspond to unprocessed proteins. By immunofluorescence, a faint and diffuse staining was observed in all the epidermal layer of a normal skin, however, additional staining were detected in psoriatic lesions (11/13, 84%). All the basal cells at the bottom of the epidermal ridges contained a dotty staining in the cytoplasm which could be observed in the juxtalesional psoriatic skin (6/13, 46%) and unfrequently observed in the non lesional psoriatic skin (1/13, 8%). These results also suggested that this expression could precede the occurrence of the clinical lesion. In reconstituted epidermis from normal and psoriatic skin biopsies, we observed the same pattern of expression by immunofluorescence as *in vivo*. By western-blot, no additional band was detected in the protein extracts of these cultures, but the intensity was five time higher. Finally, circulating epitopes (29 kDa corresponding to the mature protein) were detected in sera of normal individual and psoriatic patients. The amount of antigen was three times higher in psoriatic patient than in control. These results suggest that human endogenous retroviruses are expressed in the epidermis and that abnormal expression occurs in psoriatic lesion. The characterization of the protein recognized by these sera would help to further investigate its role in the pathogenesis of psoriasis.

187

Heterogeneity of Genetic Mutations in Erythrokeratodermas

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The erythrokeratodermas represent a group of disorders characterized by the presence of fixed or slowly moving erythematous hyperkeratotic plaques. Erythrokeratoderma variabilis (EKV; MIM: 133200) is an autosomal dominant disorder presenting with fixed, geographic plaques on extensor surfaces and transient figurate red patches at other sites. Progressive symmetric erythrokeratoderma (PSEK; MIM: 602036) is also inherited as an autosomal dominant condition with incomplete penetrance and variable expressivity. Hyperkeratotic plaques are distributed symmetrically over the body, particularly on the limbs and buttocks, but also on the face. Palmoplantar keratoderma is often present. The main distinction between EKV and PSEK is the presence of variable erythema in EKV. Mutations in the genes encoding the gap junction proteins Cx31 and Cx30.3 (*GJB3*) have been shown to cause EKV. In addition, loricrin mutations in PSEK patients which show clinical similarities to EKV, have also been described. There are also patients with EKV who do not have mutations in Cx31, Cx30.3 or loricrin. In summary, diverse mutations result in the clinical phenotype of erythrokeratoderma. Discovery of new mutations may result in a new classification of these disorders according to their genetic defects.

188

Genetic Analysis of a Severe Case of Netherton-Syndrome and Application for Prenatal Testing

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Netherton Syndrome is a rare autosomal-recessive disease with variable expression. It is defined by a triad of symptoms: congenital ichthyosiform erythrodermia, trichorrhexis invaginata and atopy. Here we report a case of a female baby from consanguineous parents of Turkish descent exhibited from birth on a generalised exfoliative erythroderma (GEE). The baby developed recurrent infections such as otitis media. A high serum IgE level and an aminoaciduria were detectable. Light microscopy of a skin biopsy revealed a moderate psoriasiforme acanthosis with lack of granular layer and a thin parakeratotic horny layer. Electron microscopy confirmed these findings demonstrating highly undifferentiated keratinocytes (e.g. low amounts of tonofilaments and keratosomes, no keratohyalin granules) that were suggestive of a severe epidermal barrier defect. At the age of 2 months hair shafts had signs of torsion nodes resembling trichorrhexis invaginata. The baby continued to suffer from recurrent septicaemia and despite of maximal intensive care support the baby died at the age of three month due to multiorgan failure secondary to a candida sepsis. Recurrent severe infections and fatal clinical courses are reported for NS especially when GEE is present at birth. Recently genetic linkage has been established to the SPINK5 gene locus on chromosome 5q32 encoding the serine protease inhibitor LEKTI. In our case DNA-sequencing of the corresponding gene identified a recurrent homozygous mononucleotide deletion (153delT) resulting in a frame shift that leads to the occurrence of a premature termination codon six residues downstream of the mutation. As expected both parents were heterozygous for the mutation. In a new pregnancy of these parents the DNA extracted from amniotic fluid had a heterozygous genotype suggesting that this foetus was phenotypically normal, and subsequently indeed a healthy baby was born.

189

TGk Mutations in a Hungarian Patient with Lamellar Ichthyosis

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Lamellar ichthyosis belongs to the genetically heterogenous group of autosomal recessive ichthyosis (ARCI). Keratinocyte transglutaminase (TGase I) mutations have been well documented in this clinical subgroup of the disease. We report a 6-year-old-girl, daughter of healthy, nonconsanguinous parents. She has thick, brown adhesive scales on the whole integument and the hairy scalp, sparing the centrofacial region. Ultrastructural analysis proved large rectangular clefts in groups and also disseminated in the stratum corneum, corresponding congenital ichthyosis type II, indicating Tgase I mutations. All 15 exons of the TGase I gene have been amplified and heteroduplexes were analysed using conformation sensitive gel electrophoresis. Bands of altered mobility were directly sequenced. We detected two known mutations (A/G substitution in the acceptor splice site of intron 5 and a G/C transversion in codon 379 of exon 7) in a compound heterozygous pattern.

190

Novel and Recurrent Mutations in the Genes Encoding Keratins K6a, K16 and K17 in 13 Cases of Pachyonychia Congenita

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Thirteen patients with pachyonychia congenita types 1 and 2 (PC-1, PC-2) were studied, two of which had a family history of pachyonychia and 11 of which were sporadic cases. Heterozygous missense or small in-frame insertion/deletion mutations were detected in the genes encoding keratins K6a, K16 and K17 in all cases. Three novel mutations, F174V, E472K and L469R were found in the K6a gene. Two novel mutations, M121T and L128Q were detected in K16. Similarly, three novel mutations L95P, S97del and L99P were found in K17. In addition, we identified recurrent mutations N171del (three instances) and F174S in K6a and R94H in K17. Analysis of both phenotype and genotype data led to the following conclusions: (a) K6a or K16 mutations produce the PC-1 phenotype whereas K17 (or K6b) mutations cause PC-2; (b) the presence of pilosebaceous cysts following puberty is the best indicator of PC-2; (c) prepubescent patients are more difficult to classify due to the lack of cysts; and (d) natal teeth are indicative of PC-2 although their absence does not preclude the PC-2 phenotype. This study establishes useful diagnostic criteria for PC-1 and PC-2, which will help limit unnecessary DNA analysis in the diagnosis and management of this genetically heterogeneous group of genodermatoses.

191

Three Novel Keratin 14 Gene Mutations in Dowling-Meara Type Epidermolysis Bullosa Simplex

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In Dowling-Meara type epidermolysis bullosa simplex mutations in genes K5 and K14 encoding the keratin intedmediate filaments expressed in basal cells have been identified. In three Hungarian families with skin fragility and palmoplantar hyperkeratosis ultrastructural studies confirmed the diagnosis. Elderly patients displayed milder, younger more severe skin symptoms. We scanned genomic sequences of keratin 14 gene in three probands. In all cases novel mutations (N123K, R125G, V133L) in helix 1 A were detected. All three mutations located in the evolutionarily highly conserved residue of the critical amino-terminal end in the rod domain. These mutations add to evidence of phenotype-genotype correlation in epidermolysis bullosa simplex, however, an explanation for the accompanying palmoplantar hyperkeratosis in Dowling-Merara disease is still missing.

192

Spontaneous Differentiation-Related Dissociation of Skin Fragility Syndrome Keratinocytes in Emerged Cultures

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Skin fragility syndrome (SFS) is an autosomal recessive genodermatosis due to mutations in the plakophilin 1 gene. Our adult patient has been previously followed up as an atypical case of Netherton syndrome. He presented with curly, sparse hair, transgedient fissurated palmo-plantar keratoderma, nail dystrophy and numerous nummular hyperkeratotic and inflammatory lesions of the skin appearing mainly at regions subjected to a moderate trauma. Skin biopsy was taken from the normally –appearing skin and subjected to biochemical, bio-molecular and immunocyto-chemical analysis. No detection of plakophilin 1 was possible using two monoclonal antibodies directed to this desmosomal plaque protein but plakoglobin, desmoplakin and desmosomal cadherins were still present in the epidermis. Desmosomes were infrequent and of small size, poorly attached to the keratin cytoskeleton. Sequencing of the RT-PCR products obtained from the extracted mRNAs confirmed the homozygous mutation of the PKP1 gene diagnosed in J. McGrath's laboratory (London, U.K.) on a sample of the patient's genomic DNA. The isolated keratinocytes were subcultured, first on 3T3 fibroblasts then in low-calcium KSFM. The cells formed little colonies and tended to disperse individually at the periphery. When seeded on dead desepidermised human dermis and allowed to stratify in immersed conditions, the cultured keratinocytes reconstructed a multilayer epithelium which would split horizontally soon after lifting at the air/medium interface. Internal tensions occurring in the upper epidermal layers due to formation of the stratum corneum apparently provoked spontaneous dissociation, not observed in normal or Hailey-Hailey control cultures.

193

Mutational Spectrum of the Δ8-Δ7 Sterol Isomerase Gene and Extent of Biochemical Defects in Patients with the Conradi-Huenermann–Happle Syndrome

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The Conradi-Huenermann-Happle (CHH) syndrome is caused by mutations in the Δ8-Δ7 sterol isomerase (emopamil binding protein) gene which result in a metabolic defect of cholesterol biosynthesis. So far, only a small number of patients have been studied world wide. We wanted to know whether there is a mutation hot spot and how often somatic and gonadal mosaicism occurs and whether the extent of biochemical alterations correlate with specific mutations. Therefore we analysed the mutations in the Δ8-Δ7 sterol isomerase gene in 16 families with the CHH syndrome, 9 of them previously reported and 7 newly investigated. Using gas chromatography and mass spectrometry (GC-MS) we also studied the accumulations of cholesterol precursors (8-dehydrocholesterol and cholest-8(9)-en-3β-ol) in serum of patients and relatives. Among the new families no further family with evidence for somatic or gonadal mosaicism was found. We observed a wide spectrum of mutations involving all four exons and no mutational hot spot. In the newly studied families we found the following novel mutations W47X, W82C, Y11X, a deletion in exon 4 and an intronic mutation involving the splice site between exon 3 and intron 3. Sterol accumulation in the serum proved to be very valuable for rapid confirmation of the diagnosis, but their extent did not correlate well with specific mutations. We conclude that there is a wide mutational spectrum of the Δ8-Δ7 sterol isomerase gene in CHH syndrome patients and altogether found 14 different mutations in the 16 families. In one family diagnosis of CHH syndrome was confirmed by typical GC-MS profile, but the mutation could not be resolved.

194

Palmoplantar Keratoderma Type Bothnia: Candidate Gene Analysis of the Disease Locus Region on Chromosome 12q13

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We have genetically characterised three types of PPK in the northern part of Sweden, of which Palmoplantar Keratoderma type Bothnia (PPKB) is the most common. PPKB is very common in the northern part of Sweden and is actually the most common genodermatose in this part of the country. PPKB is inherited as an autosomal dominant trait and is a diffuse nonepidermolytic PPK, frequently complicated by dermatophyte infections. The disorder shows strict site specificity, the skin of the patients is normal, except for the hands and feet, and there are no associated malformations or malignancies. We have mapped the disease locus to a 1.5-cM region on chromosome 12q13, using genotype data from 296 individuals from 15 families with family members diagnosed with PPKB. To date, this region on chromosome 12q13 is known to harbour at least 31 genes, however, none of these genes constitute obvious candidates for PPKB. We have decided to begin our investigations of possible candidate genes with one of the genes in the 12q13 region; the SMARCD1 gene (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 1). It has been suggested that the SWI/SNF complex is involved in chromatin-remodeling and that tissue specific chromatin-remodeling complexes may also play a role in maintenance or differentiation of diverse cell types. In addition it has been implied that the transcription of specific genes are thought to be chromatin-remodeling dependent. Therefore SMARCD1 could be a candidate for PPKB since the hyperkeratosis could be a result of distorted regulation of differentiation of the keratinocytes in epidermis of palmoplantar skin. The coding regions of the SMARCD1 gene have been analysed by denaturing-high-performance-liquid chromatography (DHPLC) and identified polymorphisms have been sequenced. So far, no polymorphism found in the SMARCD1 gene segregates with the disease.

195

Linkage to the Wiskott–Aldrich Syndrome Gene in Swedish Patients with Atopic Dermatitis

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Atopic dermatitis is a multifactorial disorder and most likely caused by common polymorphisms in many different genes in combination with several environmental factors. In the search for genetic explanations to atopic dermatitis, the clinical similarity between atopic dermatitis and the eczema manifestation in patients with Wiskott–Aldrich syndrome (WAS) made the previously identified WAS gene (located on chromosome Xp11.23) an interesting candidate gene for atopic dermatitis. We studied linkage and association to the WAS gene using four microsatellite markers in 361 Swedish families with at least two siblings affected with atopic dermatitis (in total 1325 individuals and 824 affected siblings). In the analyses we followed two different qualitative traits: atopic dermatitis and elevated allergen-specific serum IgE antibodies, and one quantitative trait, a severity score of atopic dermatitis. We found positive linkage with a maximum lod score (MLS) of 1.68 ($p \leq 0.05$) to marker MAOB following the trait severity score of atopic dermatitis. No association could be seen to either of the traits in this region using the transmission/disequilibrium test (TDT). Our results indicate that either the WAS gene or another gene in the area contributes to the severity of the atopic dermatitis phenotype in this material.

196

The Association between HLA-DR, DQ Antigens and Vulvar Lichen Sclerosus in UK

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Preliminary studies showed that certain HLA class II allotypes predominant in lichen sclerosus, implicating an immunogenetic role in its susceptibility and pathogenesis. To confirm this hypothesis, we performed DNA typing for HLA-DR and DQ antigens in the largest ever cohort of vulvar lichen sclerosus. The patients (both adults and children) were Caucasian females from the Oxford area, UK. 179 blood DNA samples from the patients were typed using the well established sequence specific primer PCR phototyping method, and the gene frequencies for DR1, DR10, DR103, DR11, DR12, DR13,DR14, DR15, DR16, DR17, DR4, DR7, DR8, DR9, DQ2, DQ4, DQ5, DQ6, DQ7, DQ8, DQ9 and DQ3 were compared with that of 602 consecutive cadaveric donor from UK (mainly local to Oxford) using 2-tailed Fisher's exact test. DR12 was present in 10.92% of patients compared with 2.68% of controls ($p < 0.0005$ with Benferroni adjustment), DR15 in 16.3% of patients v.s 30.18% of controls ($p < 0.005$), DR17 in 12.10% of patients v.s 28.04% of controls ($p < 0.05$), DR7 in 43.22% of patients v.s 29.02% of controls ($p < 0.05$); DQ7 was present in 49.02% of patient v.s 34.54% of control ($p < 0.005$ without adjustment), DQ3 in 85.96% of patients v.s. 65.06% of controls ($p < 0.01$). Other allotypes in the patient group showed no statistical differences with the control group. Our results confirmed the previous reports that DQ7 is predominant in lichen sclerosus. There seems to be strong evidence for a role of DQ7 (tested alleles include DQB1* 0301/0304/0309/0310), DR12 and DR7 in susceptibility to lichen sclerosus, whilst DR15 and DR17 may have a role in protection from lichen sclerosus.

197

Polymorphisms of the TNF Region are Associated with Pustulosis Palmaris et Plantaris Among Japanese

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We previously reported that *pustulosis palmaris et plantaris* (PPP) is associated with polymorphism of the *TNFB* gene but not the *TNFA* gene among Japanese. To identify the susceptible gene for PPP, we further determined the frequencies of various gene markers with the *TNF* region. To avoid the heterogeneity of the disease, we checked the tonsilar provocation test in all patients. According to the results, the patients group ($n = 93$) was divided into two groups (48 patients with negative result, PN and 50 patients with positive result, PP). Detection of alleles of the TNFa, TNFc, TNFd microsatellites by the PCR- Simple Sequence Length Polymorphism method revealed that the frequency of TNFd4 is decreased in PN when compared with PP or control group ($n = 247$, $p < 0.05$). However, there are no differences in the frequencies of the TNFa and TNFc alleles among the patients groups and controls. To identify the telomeric end of the susceptible locus, we further determined SNP of the *TNFB* and *TNFA* gene. Detection of polymorphism in intron 1 of the *TNFB* gene (*TNFB/NcoI* I) revealed that the frequency of allele 2 of the *TNFB* gene was increased only in PN but not PP when compared with control ($n = 67$, $p < 0.05$). Analyses of polymorphims in the *TNFA* gene revealed that the frequency of allele A (−863C, −857C) was increased in PN when compared with PP ($p < 0.05$) or controls ($n = 575$, $p < 0.0005$). Moreover, the frequency of allele B (−863 A) was decreased in PN when compared with PP ($p < 0.05$) or controls ($p < 0.01$) and the frequency of allele D (−857T) was decreased in PN when compared with controls ($p < 0.05$). To consider environmental factors for PPP, we set up another control, chronic tonsillitis; There are any differences in the frequencies of SNP of the *TNFA* and *TNFB* gene between PN/PP and chronic tonsillitis. In conclusion, PN is associated with the *TNF* region among Japanese, suggesting that the PN susceptible gene is located between TNFd and *TNFB*, and *TNFA* may be the strong candidate.

198

Interactions of Oncogenes, Tumor Suppressor, Mismatch Repair and Apoptosis-Related Genes in the Development of Melanoma

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Several genetic alterations have been implicated in the development of malignant melanoma, but the expression of oncogenes, tumor suppressor, mismatch repair and apoptosis-related genes and their interactions in melanoma have not been completely clarified. We simultaneously examined the expression of p73, c-erbB-2, ras, p53, Mdm2, p27, DCC, hMLH-1, hMSH-2, bcl-2, Bax and NF-κB, by immunocytochemistry, in both primary and metastatic melanoma cell lines derived from melanoma patients. The results showed that p73 was expressed in 7/8 cell lines, but stronger expressed in the metastatic cells than in the primary melanoma cells. c-erbB-2 was detected in all 8 cell lines and ras in 2/5 metastases. p53 was found in all the cell lines and Mdm2 in 1/8 of the cell lines. In the same patient, the intensity of p27 expression was decreased from the primary to the metastatic tumors. bcl-2 was expressed in all the cell lines. Bax was absent in 5/8 cell lines. In the same patient, Bax was weakly expressed in the primary tumor but lost in the metastases. The data demonstrate that overexpression of p73, c-erbB-2, p53 and bcl-2, and loss of Mdm2 and Bax may interact and play important roles in the development and aggressiveness of human melanoma.

199

Detection of Clonal κ Light Chain Gene Rearrangements in Cutaneous B-Cell Lymphomas by Polymerase Chain Reaction

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The polymerase chain reaction (PCR) technique has been used to detect clonal B cell populations in cutaneous biopsy material. Several studies have established PCR-based methods for the detection of immunoglobulin heavy chain (IgH) gene rearrangement using consensus primers to the VH and JH regions. Recently, Igκ gene rearrangement analysis has been utilized for the clonal analysis of B-cell lymphomas. Studies have shown that in all Igκ B-cell lymphomas and in a very large majority of Igλ B-cell lymphomas, either productive or nonproductive Igκ products are formed Therefore, the rearranged genomic products of Igκ represent a marker for B-cell clonality analysis. In this study we have utilised a single pair of consensus Vκ and Jκ primers to detect clonal κ light chain rearrangements in a panel of well-characterized cutaneous B-cell lymphomas (CBCL). DNA was isolated from frozen tissue blocks of 12 cases of CBCL and amplified using a single pair of Vκ and Jκ consensus oligonucleotides. B-cell amplicons were analyzed by means of a nondenaturing polyacrilamide gel method. Using this approach Igκ clonal products could be detected in 3 out of 12 cases of CBCL (25%). This percentage is lower than that demonstrated using oligoprimers recognizing consensus VH and JH regions (about 50%). The lack of PCR products in most of the samples investigated could be a result of several reasons. It is possible that some genes of the Vκ and Jκ families analyzed may not be efficiently amplified with the Vκ and Jκ consensus primers. However, the fact that clonal κ light chain gene rearrangements were successfully amplified in 25% of CBCL shows that a PCR analysis is reasonable with the single pair of primers used and may be used as an adjunct to IgH analysis.

200

Monocytes from Patients with Extrinsic and Intrinsic Atopic Dermatitis Exhibit Distinct Properties

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Atopic diseases are multifactorial conditions where humoral and cellular immunological mechanisms play a pivotal role in mounting allergic reactions. Particularly in atopic dermatitis (AD), it has been reported that monocytes display substantial abnormalities. A majority (70%) of the AD patients show high concentrations of total and allergen-specific IgE in blood and skin; this form has been individualized as the extrinsic AD. In the so-called intrinsic form (30%) patients have normal total serum IgE and no detectable allergen-specific IgE. This suggests that elevated allergen-specific IgE levels are not a prerequisite in the pathogenesis of at least the intrinsic form, key mechanisms contributing to the state of disease being therefore still unknown. The present study was designed to analyze in details monocytes from atopic individuals including allergic rhinitis (AR) ($n = 26$), extrinsic (EAD) ($n = 37$) and intrinsic (IAD) ($n = 7$) forms of AD under different aspects and to compare these cells with those from normal nonatopic donors (NA) ($n = 41$). Our data show that monocytes from atopic individuals constitutively express significantly higher amounts of the costimulatory molecule CD40. Additionally, the high affinity receptor for IgE (FcεRI) and the low affinity receptor for IgE (CD23) were significantly up-regulated in AR and EAD but not in IAD and correlated with the expression of the α-chain of the IL-4R (IL-4Rα; CD124). IL-4Rα was increased in AR and EAD but not in IAD patients and nonatopic controls. Since the pathophysiological background of IAD is completely elusive, these significant differences in the phenotype of monocytes from patients with EAD and IAD might be an important aspect in the investigation of the immunoregulatory mechanisms involved in the pathogenesis of IAD. Additionally our data might contribute to the development of a diagnostic tool which could enable us to distinguish between different atopic diseases and nonatopic individuals by simple monocyte-phenotyping.

201

Baff, a Ligand of the TNF Family Expressed by Dendritic Cells, is Involved in T Cell Activation

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In an effort to identify new molecules that could be used in the formulation of vaccine for cancer immunotherapy, we screened molecules expressed in Dendritic Cells for their immunostimulatory capacity. Among ligands of the TNF family, BAFF is a recently described member expressed in dendritic cells with a potent stimulatory activity on B cells. Interestingly, we found that BAFF induces the proliferation and cytokine secretion of purified human T cells in the presence of a suboptimal concentration of antibodies to the TCR/CD3 complex. BAFF is additive to the CD28 pathway. Resting T cells ($CD4^+$ and $CD8^+$) as well as primed T cells were found to be responsive to BAFF. Our data shows that BAFF is a new T cell costimulatory molecule and indicates that this TNF ligand may not only regulate humoral but also cellular immunity. BAFF may therefore be a valuable molecule in the formulation of cancer vaccine.

202

Bystander Cytolysis of $CD4^+$ T Cells as a Potential Mechanism for $CD8^+$ Effector T Cell Dominance in Contact Hypersensitivity

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For many allergic reactions to haptens and metal ions it has recently been shown that $CD8^+$ Tc1 cells rather than $CD4^+$ Th1 cells are the crucial effector cells. We have used the murine model of contact hypersensitivity (CHS) to the hapten trinitrophenol (TNP) to analyse the mechanisms underlying the predominance of $CD8^+$ T cells as effector cells. When mice were sensitized by hapten painting with trinitrochlorobenzene (TNCB) or by i.d. injection of TNP-modified dendritic cells (DC) an efficient ear swelling response was detected following ear challenge with TNCB. The effector cells isolated from local draining lymph nodes were cytotoxic, IFN-γ producing $CD8^+$ Tc1 cells. Despite the presence of immunogenic TNP-epitopes for $CD4^+$ T cells on the antigen presenting cells (APC) we did not observe $CD4^+$ T cell priming using ELISPOT assays. In an *in vitro* T cell priming system using DC as APC and T cells and APC from Perforin-deficient and Fas/FasL-deficient mice, we could show that soluble factors do not play a role in the preferential $CD8^+$ T cell priming but that the $CD8^+$ Tc1 effectors lyse the $CD4^+$ T cells in a Fas dependent manner. In addition, we found that $CD4^+$ T cells can efficiently acquire TNP from antigen presenting cells. Whether the passively acquired antigen is functionally presented on the $CD4^+$ T cell is currently under investigation. We conclude from our results that bystander cytolysis of $CD4^+$ T cells could be the mechanism underlying the preferential $CD8^+$ T cell activation observed for a number of allergic responses to haptens.

203

Intracellular Preformed DPIV/CD26 can be Mobilized Rapidly to the Lymphocytic Cell Surface: Possible Pathogenic Significance for the Atopic Disease Spectrum

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The membrane bound protease dipeptidyl peptidase IV (DPIV/CD26) cleaves neuro-peptides, growth factors, β-chemokines or HIV-1Tat. It is implicated in the pathogene-sis of autoimmune disorders. Surface levels are known to be elevated in PBLs of patients with atopic dermatitis (AD) and on birch pollen-specific T cell clones. Recently, we demonstrated a severe transport defect for intracellular proteins in PBLs of atopic pati-ents, namely a hyperreleasability of perforin-granules from cytotoxic T cells [A. Ambach et al. *J Allerg Clin Immunol* 2001 in press]. Thus, the elevated DPIV/CD26 surface levels on PBLs of atopic patients might be due to a disturbed transport mechanism for preformed DPIV/CD26. To verify this hypothesis we now investigated, if such an intracellular pool of DPIV/CD26 may exist, and if it can be mobilized rapidly to the cell surface. Ficoll isolated PBLs of healthy donors and the natural killer cell line YT were stained with anti-CD26 or anti-CD63 antibodies. Surface staining and intracellular staining was determined separately. Release of intracellular storage organelles was induced by phor-bol 12-myristate 13-acetate (PMA) and ionomycin (ION). At various timepoints cells were stained with or without saponin-permeabilization and analyzed in a flow cytometer. There was a strong intracellular staining for DPIV/CD26 in the majority of PBLs and YT cells. In addition, DPIV/CD26 surface staining increased after PMA/ION-stimulation with a time kinetic similar to that found for the secretory lysosome marker CD63. In summary, evidence is presented for the existence of an intralymphocytic pool of preformed DPIV/CD26 which can be translocated rapidly to the plasma membrane following cell activation. Thus, disturbances in the DPIV/CD26 surface level might be understand-able through the known hyperreleasability for intracellular granula in atopic lymphocytes.

204

The Immune Response of Specific T-Cell Hybridomas on Stimulation with Synthetic Complete Antigens

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Allergic contact dermatitis is mediated by a T-cell response to a hapten-modified peptide. However no endogenous complete allergen has been chemically defined. In this study we have used a pentadekapeptide, a fragment of collagen II with known affinity to MHC II, as carrier peptide. This peptide was modified by addition of haptens. The dinitrophenylmodified peptide was used for immunisation of mice. Specific T-cells were isolated and T-cell hybridomas were produced. The hybridoma cells were then stimulated by different hapten-modified peptides and the immunologic response was recorded as the thymidine incorporation. Several clones of specific T-cells were isolated, all with a strong specificity for the complete antigen used at immunisation carrying the hapten on a lysine moiety of the carrier peptide chain. No response was achieved when haptens were added to the sulfhydryl group of homocysteine which were allowed to substitute lysine in the carrier peptide. A specific but much weaker response was obtained when L-lysine was substituted to D-lysine in the carrier peptide. Neither the unsubstituted carrier peptide nor this peptide modified with a completely different hapten gave any response in any of the hybridomas. The results show the specificity of the formed T-cells hybridomas. We found a dramatic change in the immunologic response when the amino acid moiety carrying the hapten was shortened in length and the reactive group of the amino acid was changed from amino to sulfhydryl. The stereochemical requirements were of importance for the strength of the response. This immunologic model is generally useful in studying cross-reactivities.

205

Instruction of a Th1 Inducing "DC1" Phenotype and Resistance to Leishmania Major in Susceptible BALB/c Mice with Interleukin 4

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Resistance and susceptibility to intracellular pathogens are regulated by interleukin (IL-) 12, which mediates Th1 responses and resistance, and IL-4, which induces Th2 cells and susceptibility. Thus, inhibition of IL-4 or injection of IL-12 can induce Th1 responses and resistance in otherwise susceptible BALB/c mice infected with the intracellular parasite *Leishmania major* (*L. major*). Here we show that, paradoxically, IL-4 can instruct dendritic cells (DC) to become a DC1 phenotype that induces T helper cell differentiation toward a Th1 phenotype. Most importantly, priming BALB/c mice with IL-4 during infection of DC by *L. major* and prior to the activation of *L. major*–specific T cells, converted the susceptible BALB/c mice into a resistant phenotype. IL-4 primed BAPLB/c mice controlled *L. major* as efficiently as naturally resistant C3H mice and developed Th1 responses. Anti-IL-12 abrogated IL-4 induced Th1 development and resistance to *L. major*. Since immune responses evolve via the consecutive activation of first DC and, subsequently, T cells, the contrasting effects of IL-4 on DC (induction of Th1 inducing Th1 cells) and T cell differentiation (induction of Th2 cells) can lead to the maturation of T cells with opposing functional phenotypes. These findings may impact on the design of cytokine-based immune therapies currently studied in humans with melanoma or psoriasis.

206

Fumaric Acid Ester Induce Selective Reversal of the Interferon γ/Interleukin 4 Ratio in Peripheral Blood CD4⁺ T Lymphocytes

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Psoriasis is an inflammatory T cell mediated autoimmune disease dominated by interferon Error! Reference source not found. (IFN-γ) producing type 1 T cells infiltrating skin lesions. A predominant Th1 response is also found in the peripheral blood lymphocytes of the patients. Systemic therapy with dimethylfumarate is an effective treatment of psoriasis in a large number of patients but the mechanisms are still unclear. Here we investigated the *in vivo* effects of fumaric acid esters (FAE) on peripheral T and B lymphocytes during the therapy of chronic psoriasis. Eighteen patients were treated with FAE according to a standard regimen. During the first weeks oral FAE did not exceed 165 mg per day. Subsequently, the dose of FAE was increased individually in function of the subjective tolerability and the clinical improvement of psoriasis (mean of 645 mg FAE per day at 2 months of therapy). Serum and freshly isolated peripheral blood mononuclear cells (PBMC) were analyzed at regular intervals for up to 12 months. Total leukocyte counts and their relative distribution of leukocyte subsets remained largely unaffected, except for a significant increase of the eosinophil fraction between week 4 and 6 of therapy and a 7% decline of the total lymphocyte fraction. The decline in total lymphocytes was not significant during therapy. The percentage of $CD3^+$ T cells, the CD4/CD8 ratio and the CD19 cell fraction remained unchanged. Neither CD23 expression on either monocytes or B cells, nor serum IgE levels were influenced by the therapy even though FAE can induce Th2 type cytokines *in vitro*. Intracellular cytokine analysis of freshly isolated, mitogen stimulated T cells showed a significant *in vivo* increase in interleukin (IL-) 4 producing $CD4^+$ T-cells around week 6 only. Subsequently the fraction of IFN-γ expressing T cells decreased significantly in the $CD4^+$ but not in the $CD8^+$ subset. This resulted in a significant reduction of the IFN-γ/IL-4 ratio within the $CD4^+$ T cell fraction only. IL-2 and TNF-α production by $CD4^+$ T-cells remained unchanged. Cell proliferation in response to lectin mitogen or recall-antigen (tetanus toxoid) was not affected by the FAE therapy. Importantly, the peripheral suppression of IFN-γ producing $CD4^+$ cells was accompanied by a marked decrease of PASI (> 70%). Thus FAE induce a Th1/Th2 shift obviously by suppressing inappropriately activated Th1 subsets and may thus ameliorate inflammatory autoimmune diseases like psoriasis.

207

Carbamazepine-Specific T Cells are Detected in Drug-Allergic Patients after Metabolic Bioactivation by Peripheral Blood Mononuclear Cells

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Detection of drug-specific lymphocytes in patients with drug allergies is often unsuccessful. We aimed to see if peripheral blood mononuclear cells (PBMC) could metabolise carbamazepine (CBZ) to a form able to elicit lymphocyte proliferation and to use our novel 2-phase lymphocyte proliferation assay to characterise the responding lymphocytes. 13 patients with allergy to CBZ (erythema multiforme) and 7 patients taking the drug with no problem were tested. PBMC were incubated with a range of concentrations of CBZ in flat-bottomed wells for 48 h. The supernatants were removed and filtered and the remaining adherent cells washed. Fresh PBMC were added to both the supernatant and the adherent cells for 6 days. Proliferation, detected as ^{3}H-thymidine uptake, was expressed as stimulation index(SI), compared with antigen-free cultures. All 13 CBZ-allergic patients gave SI greater than 3 (median 20, IQ range 5–95) when cells were culture in supernatants; cells cultured with the adherent macrophages or in conventional lymphocyte proliferation assays failed to respond; the nonallergic patients all gave negative responses. Analysis of the responding lymphocytes ($N = 4$) showed they were $CD4^+$ and secreted predominantly IL-5 up to (1100 pg per mL) with small amounts of IFNγ. Addition of ketoconazole (30microM) during the 48 h incubation, reduced the subsequent lymphocyte SI from a median of 30–6 ($N = 4$) suggesting CYP450-mediated metabolism is required. This novel assay allows metabolic bioactivation of native drugs to generate metabolites recognisable by T cells *in vitro*. The system also allows analysis of the T cell phenotype and the metabolic pathways involved in bioactivation.

208

Differential Effects of Tuberculin and Sodium Lauryl Sulphate on Impedance Response Patterns in Human Skin

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When an electrical potential is applied to human tissue, the pattern of the resulting current flow is determined by shapes, arrangements, and internal structure of tissue cells. By measurement of the electrical current patterns over a range of frequencies, well-defined indices describing the tissue structure can be calculated. In this study, we used a pencil probe (diameter 10 mm) to measure impedance spectra on skin reactions induced by 0.1 mL of PPD (2TU) that was injected intracutaneously on the volar forearms of 16 adult healthy subjects, all known to be tuberculin-sensitized. Assessments were performed 3 and 7 days after the injections, using visual scoring, a new electrical impedance technique and transepidermal water loss. Compared to relevant controls and reactions induced by sodium lauryl sulphate (SLS), significant changes in the impedance parameters were found. Unlike the irritant reactions, no significant increase in transepidermal water loss values in tuberculin reactions were found. This can be explained by the fact that the epidermis is little affected, since the inflammatory process in the tuberculin reaction is located deeper in the dermis than with the irritant reaction. Characteristics of the electrical impedance spectra may be explained by changes in cellular and intracellular events, and the current study suggests that electrical impedance in the described implementation may be used to give good separation of PPD- and SLS-induced skin reactions.

209

Irritant and Allergic Contact Reactions Assessed by Electrical Impedance

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When an electrical potential is applied to human tissue, the pattern of the resulting current flow is determined by shapes, arrangements, and internal structure of tissue cells. By measurement of the electrical current patterns over a range of frequencies, well-defined indices describing the tissue structure can be calculated. We used a pencil probe (diameter 10 mm) to measure impedance spectra of nickel allergic contact reactions and irritant contact reactions induced by sodium lauryl sulphate (SLS). Various concentrations of the test substances in distilled water were applied on the volar forearms of 19 adult women, all known to be nickel allergic. Assessments were performed 3 and 7 days after the applications. Measurements made on the two types of cutaneous reactions were very well separated. Compared to the reactions induced by SLS, statistically significant changes in three of the four impedance indices were found on day 3: namely the means of index MIX and index IMIX ($p \leq 0.001$) and the mean of index RIX decreased ($p \leq 0.01$). Characteristics of the electrical impedance spectra may be explained by changes in cellular and intracellular events, and the current study suggests that electrical impedance in the described implementation may be used to give good separation of contact reactions of allergic and irritant nature.

210

***In Vivo* Spontaneous Semipublic T Cell Response Against Melanoma (B16) Disappear During Tumor Rejection Induced by GMCSF**

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The numerous studies in mice and humans concerning the T cell repertoire in melanocytic tumor have resulted in conflicting data. Efficient antimelanocytic responses was found oligoclonal in spontaneous melanoma rejection and in halo-nevi in human. In contrast, the T cell response to progressing tumors is more diverse, not to say polyclonal. The present work is aimed at approaching the *in vivo* diversity of the β chains of the T-cell receptor (TCR) of the T cells present in B16 melanoma during tumoral growing and in B16 undergoing immune-mediated regression. In order to avoid biases introduced by *in vitro* cloning we have used the "immunoscope" technology that permits to describe the BV, BJ and CDR3 length usages of the T cells. C57BL/6 mice were subcutaneously injected in two sites with 10^5 irradiated B16/F10 transfected with GMCSF or with PBS as control. At day 14 following the injection mice were injected with 5.10^5 B16. All mice injected with B16/F10/GMCSF rejected B16 melanoma and were resistant to a new challenge. Spleen, lymph node, and tumoral total RNA was extracted for TcR repertoire analysis.the BV-BC PCR products prepared from non rejected B16 tumors in control mice displayed diverse patterns. Most BV-BC combinations yielded a gaussian signal indicative of an absence of specific stimulation of the corresponding T cells, whereas BV11-BJ2.1 yielded isolated peaks in 5/5 mice with a unique CDR3 length, indicative of the presence of a semi public antigen-driven T cell expansion. *In vivo* distribution of TCR β chain in mice injected with B16/F10/GMCSF showed a gaussian distribution. The expanded BV11-BJ2.1 T cell clone was not expanded in the spleen and lymph nodes of B16 resistant mice. In conclusion, disappearance of the spontaneous semipublic T cell response, and polyclonal T cell expansion are associated with the efficiency of the GMCSF induced immune regression process in B16 melanoma.

211

"Natural" Immune Responses in Melanoma Patients

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Cell responses against tumor-associated antigens (TAA) have been detected in melanoma patients following vaccination with tumor-peptides or peptide-pulsed Dendritic Cells. Apart from individual reports with limited numbers of patients, surprisingly little is known about the "natural" immune responses of nonvaccinated patients and their correlation with the course of disease. We have looked for circulatory T cells in patients with malignant melanoma (primary and metastatic) recognizing different HLA-A1, A2.1 and A3- restricted tumor peptides using the Elispot-assay. The phenotype as well as the activation- and homing marker profile where further characterized by intracellular cytokine staining or with the tetramer-technique. In the majority of melanoma patients the frequency of TAA-recognizing T cells was below the detection limit directly *ex vivo* or in frozen samples of peripheral blood mononuclear cells. After only one or two peptide-specific restimulations *in vitro* we could detect high numbers of TAA-recognizing T-cells particularly in patients with a rather favorable course of disease. By evaluating more melanoma patients at different stages or with variable courses of disease we hope to further elucidate the role of TAA-recognizing T cells in melanoma.

212

Coordinated Loss of Melanoma Differentiation Antigens in Metastatic Melanoma

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The melanoma differentiation antigens (MDA) gp100, MART-1 and Tyrosinase are involved in a common pathway of melanin synthesis and it can be assumed, that disruption of this pathway as indicated by the common occurence of amelanotic metastases might affect several of these antigens. Peptides derived from these MDAs are used in immunotherapy of melanoma and antibodies recognizing these antigens are commonly applied to detect melanocytic lesions. 191 paraffin embedded melanoma metastases from 28 patients which had between 2 and 19 (mean 6.8) lesions were analyzed by immunohistochemistry for the expression of gp100 (antibody HMB-45), MART-1 (A103) and Tyrosinase (T311), and were compared with the expression pattern of the cancer testis antigens MAGE-1 (77-b) and MAGE-3 (57-b), as well as with the highly sensitive antibodies anti-S100 and SM5-1. The overall reactivities were 81.6% (gp100), 79.5% (MART-1), 59.6% (Tyrosinase), 59.1% (MAGE-1), 60.7% (MAGE-3), 91.6% (SM5-1) and 93,2% (S100). Only 27 lesions (14.1%) were positive for all tumor associated antigens (TAA), while 75 lesions (39.2%) had lost only one antigen. 87 lesions (45.5%) were negative for several of the antigens. 9.4% of the lesions were negative for Gp100 and MART-1, 11.0% for Gp100 and tyrosinase, 15.2% for MART-1 and tyrosinase and 8.9% for Gp100, MART-1 and tyrosinase, which is up to 6 times higher than the expected calculated loss. Thus, beside a marked heterogeneity, there is a coordinated loss of MDAs in melanoma which did not include cancer testis antigens and S100 or SM5-1. Combined loss of gp100 and SM5-1 was only found in 1/191 cases (0.5%) and is thus the most valuable combination of antibodies for detecting melanoma metastases. Gp100 showed a strong average staining, while all other antigens stained moderate to strong (MART-1, tyrosinase, MAGE-3) or moderate (MAGE-1). Only MAGE-3 stained more than 75% of cells within a lesion by average, while all other antigens were expressed in 50–75% of cells. Single epitope immunotherapy will be of limited use in the majority of the cases. Even when multiple epitopes are combined, the higher than expected coordinated loss of MDAs, the low protein expression, the insufficient number of positive cells within a lesion in conjunction with other tumor escape mechanisms might be an explanation for the modest clinical effectiveness of antigen-specific immunotherapeutical approaches of melanoma.

213

Expression of CD40 and the Mitogenic Peptide Hormone Receptors Vasopressin V1 and Kinin B1 and B2 Receptors in Psoriatic Epidermis

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Keratinocytes express various receptors that once activated induce in some cases cell proliferation and in others inhibition of DNA synthesis, effects that could be relevant in the pathogenesis and/or perpetuation of skin diseases like psoriasis. Since CD40 has been involved in keratinocyte differentiation, and peptide hormones such as kinins and vasopressin are mitogenic in different cellular systems, we wonder if the psoriatic epidermis express kinin B1 and B2 and vasopressin V1 receptors and whether they are associated to CD40 expression. Binding of a specific iodinated B1 agonist (Tyr-Lys-Aca-Lys-des[Arg^9]-bradykinin) was restricted to basal layers of the lesional epidermis; whereas a B2 agonist (Tyr-bradykinin), bound the whole lesional epithelium. Normal epidermis displayed an equivalent pattern of binding but of lesser reactivity. Similar results were obtained using antibodies that recognize the B1 (Merck, and Co. Inc., USA) and B2 receptors. Interestingly, the cellular layers involved in these reactions were also immunoreactive to V1 and Ki-67. Keratinocytes of normal epidermis were less immunoreactive to V1 and Ki-67 but exhibited greater staining to CD40 than psoriatic lesions. Further, a specific biotinylated probe confirmed the expression of B1 receptor mRNA in psoriatic keratinocytes and, to a lesser extent, in normal epidermis. Future studies should elucidate the relative contribution of CD40 and mitogenic peptides to both keratinocyte proliferation and differentiation disorder, and/or to the inflammatory process in psoriasis.

214

Tissue Transglutaminase is Overexpressed in Psoriasis and Palmoplantar Pustulosis

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Several transglutaminases (TG) are expressed in the skin, e.g. keratinocyte TG (kTG or TG 1) in the granular layer in normal skin and Factor XIIIa in the dermis. In psoriasis lesions the normal kTG pattern is replaced by a staining of the upper part of stratum spinosum and Factor XIIIa is highly overexpressed in the papillary dermis. Tissue transglutaminase (tTG or TG 2) has recently been identified as the main autoantigen in coeliac disease. tTG is associated with angiogenesis and there is an increased expression/activity in wound healing. Subgroups of patients with psoriasis and palmoplantar pustulosis (PPP) have antibodies to gliadin but most of them have no antibodies to endomysium/transglutaminase. In PPP there is an increased prevalence of coeliac disease. Psoriasis and PPP patients with antibodies to gliadin improve on gluten-free diet but the mechanisms are unknown. The aim of this study was to compare with immunohistochemistry the expression of tissue transglutaminase in involved and noninvolved skin in psoriasis and PPP with that in corresponding sites in healthy subjects. In normal skin, tTG was expressed in in blood vessels, the dermal part of the sweat gland and in the arrector muscle whereas the expression in the hair follicle was weak. In psoriasis lesions there was an increased staining, mainly localized to the endothelium in the papillary dermis but in many specimens all layers of the epidermis were also stained. In PPP this abnormal pattern was even more pronounced with stronger staining intensity of the endothelium than in psoriasis. The conclusion is that tTG is highly overexpressed in involved psoriasis and PPP skin. In PPP the strongest expression is in the papillary endothelium, recently demonstrated to be the target of an autoimmune reaction.

215

$CD3^+CD56^+$ NK T Cells are Significantly Decreased in the Peripheral Blood of Patients with Psoriasis

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Psoriasis is a chronic, infalmmatory, hyperproliferative skin disease, in which autoimmunity plays a great role. Natural killer T cells (NK T cells), are suggested to be involved in the pathogenesis of different autoimmune diseases. To examine the involvement of $CD3^+CD56^+$ NK T cells in the pathogenesis of psoriasis, we investigated the lymphocyte subpopulations obtained from blood samples of psoriatic patients before and after treatment, and of healthy controls, using two-colour flow cytometry. We found no significant differences between total T cells, total B cells, T helper cells, T cytotoxic cells and NK cells in patients with psoriasis before and after treatment and in controls. Increased percentage of memory T cells and decreased percentage of naive T cells was detected in patients compared to controls. Treatments used in this study had no effect on memory and naive T cells. The $CD3^+CD56^+$ NK T cells of psoriatic patients were significantly decreased in relative to controls. The percentage of $CD3^+CD56^+$ NK T cells increased after different antipsoriatic therapies, but remained significantly lower than those found in controls. $CD3^+CD56^+$ NK T cells of healthy controls were capable of rapid activation, while in psoriatic patients activated NK T cells were almost absent. The decrease in the number of $CD3^+CD56^+$ NK T cells may represent an intrinsic characteristic feature of patients with psoriasis, which is supported by the fact that after treatment NK T cells do not reach the values found in controls. In conclusion our results suggest that $CD3^+CD56^+$ NK T cells could be actively involved in the development of Th1 mediated autoimmune diseases

216

Palmoplantar Pustulosis – An Autoimmune Disease Induced by Smoking?

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The mechanisms underlying the inflammation in palmoplantar pustulosis (PPP) are not known. Most PPP patients (95%) are smokers. There is a high prevalence of autoimmune disease in PPP patients. The target for the inflammation seems to be the acrosyringium, which has lost its normal pattern. In the acrosyringium there is a strong expression of nicotinic receptors of α-7 type. We previously found antibodies (ab) to nicotinic receptors in PPP sera. Forty-seven per cent of the PPP sera showed an immunofluorescence on endothelial cells in specimens from normal palmar skin from a nonsmoker, especially in the papillary dermis. The staining intensity was stronger in PPP patients with nAChR ab than in those without nAChR ab. We wanted to study if sera from PPP patients reacted differently on palmar skin from a smoker compared to the nonsmoker. Immunofluorescense (IF) with sera from 45 PPP patients was performed on sections from palmar skin from a smoking healthy person. The immunofluorescense showed positive structures in the papillary dermis like those expressed on normal palmar skin from the nonsmoker (e.g. endothelial cells), but in addition there was also immunofluorescence in the sweat duct and gland. The interpretation is that smoking induces an up-regulation of an antigen in normal palmar skin. PPP might be an autoimmune disease and nicotine might be of importance for the start and maintenance of the disease.

217

High Affinity IgE Receptors on Antigen Presenting Cells (APC): a Therapeutic Target in Atopy?

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IgE-facilitated allergen presentation via FcεRI may be a key pathogenic mechanism whereby atopic T cells are potently activated by APC. However, the validity of the high affinity FcεRI–IgE interaction as a therapeutic target is uncertain, as prior lactic acid stripping of IgE from APC is reported as essential for antibody-mediated FcεRI blockade. In contrast, we have shown that the *in vitro* proliferative responses of atopic peripheral blood mononuclear cells (PBMC) to house dust mite (HDM) allergens in the presence of 10% fresh autologous (IgE-containing) serum may be greatly reduced by heating serum at 56°C for 30–60 min, conditions that irreversibly denature IgE (maximum stimulation index [mSI] 36 ± 30 and 12 ± 17 with fresh unheated vs. heated autologous serum, respectively; means $\pm$ SD, $n = 13$, $p = 0.003$, paired *T*-test). This suggests a possible equilibrium between IgE on FcεRI and in medium *in vitro*. Prior lactic acid stripping of atopic PBMC (pH 3.9 for 5 min) did not enhance the differences between responses in the presence of unheated autologous serum vs. heated autologous serum (mSI 37 ± 26 vs. 11 ± 22 without lactic acid stripping; mSI 18 ± 6 vs. 8 ± 8 with stripping; $n = 4$). Finally, in preliminary experiments, proliferative responses to HDM were inhibited by affinity-purified FcεRI antibody 3G6 (10 μg/mL, Upstate Biotech), whether or not the cells were subjected to prior lactic acid stripping (42% vs. 40% reduction in mSI without vs. with lactic acid stripping, respectively). Therefore, direct antagonism of FcεRI on APC may be a viable therapeutic approach in atopy.

218

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

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Cutaneous T-cell lymphomas (CTCL) are clonal proliferations of CD4+ T lymphocytes primarily involving the skin. Mycosis fungoides is an epidermotropic CD4+ CTCL, and a more aggressive form Sezary syndrome occurs when the malignant cells become nonepidermotropic. The role of neuropeptides on the growth and chemotaxis capacity of CTCL cells remains unknown. Here, we report that CTCL cells, similarly to normal resting or activated peripheral lymphocytes, were able to bind neurotensin. We used an IL-2-dependent CTCL malignant T cell line derived from CTCL lesions in order to study the role of neurotensin on the proliferation and the migration of these malignant cells. First, we determined that these malignant cells expressed neurotensin receptors on their cell membrane. Functional results indicated that neurotensin did not stimulate the growth of the cell line. In contrast, this neuropeptide inhibited the proliferation of the tumor cells in response to exogenous IL-2. Furthermore, we found that neurotensin enhanced both spontaneous and chemoattractant-induced migration of the malignant cells. These results suggest that neurotensin in skin can play a role in the evolution of the disease by locally limiting the growth of the CTCL tumor cells in response to cytokines and by enhancing their chemotaxis capacity.

219

Acquired CD40-Ligand Deficiency in Sézary Syndrome

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Sézary syndrome (SS) is a form of cutaneous T cell lymphoma that is characterized by prominent immunologic defects including depressed cell-mediated immunity and defective TH1 cytokine production. The purpose of this study was to determine if CD40L, a cytokine known to be important for TH1 responses, is involved in the pathogenesis of SS and a possible candidate for therapeutic intervention. We show by FACS analysis that CD4+ T lymphocytes of Sézary patients (7/7 cases) have a marked defect in CD40L expression upon activation by anti-CD3 *in vitro*. This defect is not restricted to the CD4+/CD7- population, and is more pronounced in patients with a high circulating tumor burden. In these patients, production of IFNγ, TNFα and IL-12 by peripheral blood mononuclear cells (PBMC) after *in vitro* T cell activation was also strongly reduced, and most markedly in those with a high tumor burden. As a similar defect was reproduced in healthy control PBMC by CD40L blockade, and no defect in CD40 expression was detected in Sézary patient's APC, defective CD40L expression and signaling was thought to be a possible cause. Indeed, incubation of PBMC from Sézary patients with recombinant soluble CD40L+ IFNγ, but not IFNγ alone, partially restored TNF α and IL-12 production. We thus propose that acquired deficiency of CD40L on CD3-activated T cells in SS accounts for some of the acquired immune defects of patients with SS. As shown here *in vitro*, recombinant CD40L may be a rational approach to therapy.

220

TCR ãä T Cells in Peripheral Blood and Skin in Systemic Lupus Erythematosus Patients

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the deposition of autoantibodies and immune complexes leading to tissue damage. Despite significant advances in our understanding of the etiopathogenesis of lupus till now the mechanisms responsible for the breakdown of immunological balance is searched. Among multiple functional defects in different cells of the immune system the role of ãä T cells in the disease pathogenesis is underlined in many reports. The purpose of our study was to evaluate the total account of ãä lymphocytes and their subpopulations in peripheral blood and skin in patients with active and inactive phase of systemic lupus erythematosus and in healthy volunteers. The investigations were performed using flow cytometry and immunohistochemical methods. Statistical analysis showed the significant decrease of the total number of ãä T cells in peripheral blood of SLE patients in both active and inactive stage of the disease comparing to the control group. The similar results were noticed in Vä2 TCR+, Vä9 TCR+, CD3+, CD4+ and CD8+ subpopulations. On the contrary the number of Vä3 TCR+ lymphocytes was only slightly elevated in SLE patients but not confirmed with statistical methods. These values were not dependent on the disease activity. Different results were obtained in skin. The total number of ãä T cells and Vä2 TCR + subpopulation were higher in SLE patients than in control group and correlated with disease activity. Vä3 TCR+ was higher, in comparison to control, only in active phase of the disease. Vä 9 TCR+ and CD8+ were increased in all SLE patients and did not differ between active and inactive stage. The number of CD3+ lymphocytes did not differ in patients and control. The total number of CD3+ lymphocytes was almost equal in SLE patients and healthy individuals. The obtained results may indicate the possible role of ãä T cells and their subpopulations in SLE pathogenesis. The differences in number of T cells between circulation and tissue may suggest their migration from blood to skin in SLE patients mainly during exacerbation of the disease. Other possible explanation might be the thesis that antilymphocytic antibodies may play a role in the depletion of this cellular subset in peripheral blood. The titer of anti-T cell antibodies could correlate with the disease activity.

221

Infiltrating Cells and Related Cytokines in Lesional Skin of Patients with Chronic Idiopatic Urticaria and Positive Autologous Serum Skin Test

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The term chronic autoimmune urticaria (CAIU) is used to identify a group of patients with chronic idiopatic urticaria (CIU) and circulating auto-antibodies (IgG, IgM) against the high-affinity IgE receptor and/or against IgE. These auto-antibodies have the capability to provoke *in vivo* a wheal and flare response in this group of patients by intradermal injection of autologous serum (autologous serum skin test, ASST), and *in vitro* to evoke mediator release from basophils and mast cells; therefore it has been suggested that these auto-antibodies might have an important role in the pathogenesis of the disease. In the present study an immunohistochemical analysis of infiltrating cells (CD4, MPO, EG1, EG2, tryptase) was performed on spontaneous wheals of 7 patients (4M, 3F; median age: 45 years; range: 23–50) with CIU and positive ASST in comparison with their uninvoled skin and with skin from 4 healthy donors. We also evaluated the presence of cytokines (IL-4, IL-5, IFN-γ) and chemokines (IL-8, CCR-3, CXCR-3) correlated to the activity of the lesional skin infiltrating cells. In the lesional skin we could observe an increased number of CD4+ T lymphocytes (29.1 ± 11.3) if compared to controls (skin from healty donors) (6.3 ± 1.3), an increased number of neutrophils (MPO: 86.1 ± 14.1 vs. 17.6 ± 1.7) and eosinophils (EG1: 23 ± 9.9 vs. 3.3 ± 3.5; EG2: 26.3 ± 6.5 vs. 2 ± 0.1) whereas mast cells did not show a significant variation ($20{,}3 \pm 6$ vs. 23.7 ± 6.06). We observed an expression of IL-4, IL-5, and IL-8 only in the lesional skin, while IFN-γ showed a slight expression in lesional skin and in uninvolded skin. CXCR-3 had a marked expression only in the lesional skin (46.6 ± 13.2 vs. 20.7 ± 7.9) whereas CCR-3 had a similar expression also in the controls. Our results document a significant role of CD4+ Th2 lymphocytes and neutrophils in the pathogenesis of both spontaneous and autologous serum-induced wheals in CIU.

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222

Mast Cells Promote Wound Healing by Initiating Inflammation

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We have recently demonstrated that murine skin mast cells (MC), key regulators of inflammation, are required for normal early wound healing: Skin wounds in genetically MC-deficient Kit^{W}/Kit^{W-v}-mice are significantly larger during the first four days after wounding as compared to normal+/+ mice or Kit^{W}/Kit^{W-v}-mice that were reconstituted with bone marrow derived cultured MC. Speculating, that induction of inflammation may be one mechanism by which MC promote wound healing, we have quantified (1) MC activation in wounded skin of C57BL/6 mice and (2) early inflammatory skin reactions to experimentally induced full-thickness skin wounds in MC-deficient Kit^{W}/Kit^{W-v}-mice and normal+/+ mice. The extent of MC-degranulation in 6 mm punch biopsy-induced back skin wounds of C57BL/6 mice was assessed by histomorphometric analysis of plastic embedded, alkaline Giemsa stained 1 μm sections of skin-biopsies obtained 1 h after wounding. Skin adjacent to wound area showed markedly more extensively degranulated MC ($66 \pm 8\%$) as compared to normal control skin ($1 \pm 1\%$, $p < 0001$). The extent of MC degranulation dropped with increasing distance from the wound area. To test whether MC-degranulation after wounding contributes to the inflammatory phase of wound healing, we have examined the extravasation of Evan's blue (EB) in early wounds of Kit^{W}/Kit^{W-v}-mice and normal *Kit*+/+ mice. *Kit*+/+ mice exhibit prominent extravasation of EB in skin adjoining wounds as compared to normal control skin (56 ± 5 ng EB/g tissue vs. 11 ± 2 ng EB per g tissue, $p < 0.001$, 30 min after wounding), significantly more ($p < 0001$) than Kit^{W}/Kit^{W-v}-mice that showed no significant inflammatory response after wounding (wound area: 16 ± 2 ng EB/g tissue vs. Control skin: 10 ± 2 ng EB per g tissue) These data point towards a crucial role of activated MC in the initial inflammatory phase of skin wound healing. Our findings suggest that MC-induced inflammation is required for normal wound healing.

223

Altered Regulation of Apoptosis in Cutaneous Mastocytosis

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Mastocytosis is characterized by a focal or generalized increase of mast cells, particularly in the skin, but also in other organs. In order to investigate whether an altered regulation of mast cell apoptosis may be involved in the pathogenesis of mastocytosis, the number of apoptotic TUNEL-positive mast cells, the proliferation marker Ki67, and the expression of the apoptosis-regulating proteins P53, Bcl-2, and Bcl-XL were studied by immunohistochemistry in cutaneous lesions of 8 infants with solitary mastocytomas, 5 children with multiple mastocytomas, 11 children with generalized urticaria pigmentosa, and 7 adults with urticaria pigmentosa and compared to skin from 7 normal controls. In addition, semiquantitative RT-PCR was used to examine the expression bcl-2 and bcl-XL mRNA in cutaneous lesions of 6 children and 3 adults with mastocytosis. The number of toluidine blue-positive mast cells was significantly increased in all patients, particularly in children. In contrast to other forms of cutaneous mastocytosis, a decreased number of TUNEL-positive mast cells was observed in solitary mastocytomas. While the expression of Ki67, P53, and Bcl-XL in mastocytosis lesions was comparable to normal skin, the expression of Bcl-2 was significantly enhanced in solitary mastocytomas and urticaria pigmentosa, particularly in lesions of less than one year duration. Accordingly, an increased expression of bcl-2 mRNA, but not bcl-XL mRNA, was found in mastocytosis lesions. These findings demonstrate that the apoptosis-preventing molecule bcl-2 is overexpresssed in cutaneous lesions of mastocytosis. Alterations in the mechanisms of apoptosis may in part account for the accumulation of mast cells in mastocytosis.

224

Toll-Like Receptors 2 and 4 are Expressed on Human Keratinocytes and Mediate the Killing of Pathogens

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Keratinocytes have the ability to kill pathogenic fungi and bacteria by producing antimicrobial compounds such as nitric oxide and antimicrobial peptides. Previously we demonstrated that keratinocytes have a direct candidacial activity, which may be mediated by the keratinocyte mannose receptor. The present study was designed to explore whether Toll-like receptors (TLRs) and activation of NF-κB could be responsible for mediating the killing of pathogenic microorganisms by keratinocytes. RT-PCR analysis was used to examine the expression of the two main pattern recognition receptors (TLR2 and TLR4) in cultured epidermal keratinocytes in the third passage and in HaCaT cells. The activation and nuclear translocation of NF-κB in cultured keratinocytes and HaCaT cells was examined by electrophoretic mobility shift assay (EMSA). We demonstrated that both unstimulated cultured keratinocytes and HaCaT cells express TLR2 and TLR4 mRNA, which could be regulated by induction of cells with pathogenic microorganisms or by cell differentiation. Incubation of cultured keratinocytes and HaCaT cells with *Candida albicans, Staphylococcus aureus, Mycobacterium tuberculosis* or the fungal cell wall component mannan, and the Gram negative cell wall component LPS, resulted in the activation and nuclear translocation of NF-κB. As the expression of inducible nitric oxide synthase (iNOS) is known to be regulated by NF-κB we examined the expression of iNOS by RT-PCR. We demonstrated that pathogenic microorganisms induce iNOS mRNA expression in keratinocytes. In our work, we give the first evidence that epidermal keratinocytes express toll-like receptor 2 and 4 and microbial compounds activate NF-κB, which may be involved in pathogen-mediated intracellular signaling.

225

The T-Cell Mitogen of Propionibacterium Acnes is a Cell Wall Component

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We have previously reported that whole cells of *P. acnes*, the microorganism associated with acne, has mitogenic activity. We are attempting to clarify, chemically characterize and locate the T-cell mitogen in the cells. This study reports on its' location in *P. acnes*. The laboratory strain *P. acnes* P37 was grown and the cells processed to give three fractions: material external to the cell wall and not covalently bound; cell-wall components; and soluble cytoplasmic components. A lymphocyte transformation assay (LTA) was used to detect responses of seven cord blood mononuclear cell (CBMNC) samples to the different fractions before and after incubation with a mouse-antihuman MHC class II monoclonal antibody (TU39), which inhibits an antigenic response to *P. acnes* products. 7/7 CBMNC samples were stimulated by cell-wall components of *P. acnes* and this reaction could not be blocked by TU39. All 7 samples were activated by the cytoplasmic cell fraction and 5 by the external cell wall fraction but with lower stimulation indices compared to the cell wall components. Furthermore, TU39 totally blocked the stimulation of 5/7 and 3/5 lymphocyte samples when exposed to the cytoplasmic and external cell wall fractions, respectively. We conclude the T-cell mitogen is located covalently bound to the cell wall of *P. acnes* and the activity shown in the other fractions to some of the CBMNC samples was due to minor distribution of the T-cell mitogen during cell breakage and partial separation combined with varying sensitivity of CBMNCs.

226

Propionibacterium Acnes Reactive T-Cell Lines Established from Early Inflamed Acne Lesions

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Recent studies have indicated that inflammatory acne is a T cell mediated disease with *Propionibacterium acnes* (*P. acnes*) being implicated as the major stimulus for acne inflammation. To determine whether *P. acnes* –reactive T cells are present in acne skin, T cell lines (TCL) were cultured from 7 skin biopsies of early inflamed lesions and tested in a proliferation assay with sonicated *P. acnes*. The TCL were stained for CD4, CD8 and TCR-αβ expression and analysed by flow cytometry. Skin biopsy fragments were placed in tissue culture medium supplemented with human AB^+ serum and sonicated *P. acnes*. T lymphocytes and dendritic cells migrated from skin biopsies and formed clusters. TCL were cultured for 10–14 days, adding Interleukin-2 every 3 days. Two of the seven TCL died off. The remaining TCL all responded to sonicated *P. acnes* (at optimal dilutions) to a varying extent. The mean counts per minute; c.p.m, ranged from 9470 to 17056. The TCL proliferated to a greater extent (17728–59849 c.p.m) using the positive control, phytohaemagglutinin (PHA). The skin TCL consisted predominantly of TCR-$\alpha\beta^+$, $CD4^+$ T cells (89.2–98.2%) with minimal (0.3–8.9%) $CD8^+$ T cells present. This is compatible with the findings of previous histological studies of acne lesions. This study shows that T lymphocytes can be cultured from early inflamed acne lesions and confirms that cellular infiltrates in acne lesions are predominantly CD4-positive. The TCL are reactive to *P. acnes*, the natural flora in pilosebaceous follicles.

227

Differences Between Propionibacterium Acnes Strains for T-Cell Stimulation

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Preliminary data showed that *P. acnes* is associated with inflammatory acne via antigenic and T-cell mitogenic mechanisms. To determine whether the stimulating capacity of two clinical isolates, one from inflammatory acne and one from a case of endocarditis, were different to the laboratory strain P37, a lymphocyte transformation assay (LTA) was performed with 11 peripheral blood mononuclear cell (PBMNC) samples from patients with different variants of inflammatory acne, as well as with 9 cord blood mononuclear cell (CBMNC) samples. Whereas 9/9 CBMNC samples were stimulated by all three bacterial isolates and showed maximal stimulation between days 5 and 7, 6/11 of the PBMNC samples were reactive with two peaks of stimulation at day 3 and 7. Maximal stimulation indices (SI) were higher for the clinical isolates in 8/9 CBMNC samples (4/6 PBMNC samples). Although statistical analysis did not reveal significant differences between the three bacterial strains in a time course experiment, total SI-values were higher for the clinical isolates compared to P37. Although mass spectrometry analysis of cells had revealed only minor differences between clinical isolates and the laboratory strain *P. acnes* P37 there may be differences between *P. acnes* isolates for their immunogenic/mitogenic capacities and/or susceptibility in patients with inflammatory acne.

228

Skin Fibroblasts Exert an Inhibitory Effect on Neutrophil Oxidative Stress

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Fairly recently it has been suggested that fibroblasts play an important role in the switch from an acute inflammation to the adaptive immunity and tissue repair. The aim of our study was to evaluate the influence of fibroblasts on neutrophil (PMNL) by assessing its production of reactive oxygen intermediates (ROI). The study was performed on two-cell *in vitro* system, i.e. human fibroblasts of the third passage obtained from the healthy skin with blood neutrophils from healthy donors. Neutrophils:fibroblasts ratio was 10 : 1 and 100 : 1, incubation temperature 37°C, in 5% CO_2, in PBS medium. PMNL ROI production (oxidative stress) was evaluated by luminol-enhanced chemiluminescence (RLU_{Total}) immediately after adding PMNL to fibroblast system [0 min], after 15, 30 and 45 min Some neutrophils were also primed with TNF-alfa (at concentration 10 ng per mL, for 15 min, at 22°C). The system was subsequently stimulated by N-formyl-methionyl-leucyl-phenylalanine (N-fMLP) at concentration 2×10^{-6} M or phorbol-myristate-acetate (PMA) at concentration 200 ng per mL. We observed 1. fibroblasts demonstrated an inhibitory effect on ROI production by nonprimed neutrophils when comparing to nonprimed neutrophiles alone when the system was stimulated by N-fMLP (mean 0.12 vs. 0.25 RLU_{Total}, respectively; $p < 0.05$), when stimulated with PMA only slight inhibitory effect was noted 2. TNF-alfa primed neutrophils to some extent abrogated the above inhibitory effect of fibroblasts after N-fMLP stimulation (mean 0.24 vs. 0.33 RLV_{Total}, respectively; $p < 0.05$); 3. the inhibition effect depends on the time of cell-cell contact (the most pronounced at time [0] and subsequently it diminishes, reaching plateau at 30 min) and the number of fibroblasts (the more fibroblasts in the system, the more pronounced blocking effect). In conclusion, it seems that skin fibroblasts exert an inhibitory effect on neutrophil oxidative stress reaction.

229

Human Sebocytes Express CD14 Molecules and their IL8 Production is Induced by Both CD14-Dependent and Independent Pathways

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We have previously reported that lipopolysaccharides (LPS) stimulate IL8 secretion by human sebocytes but do not influence IL1α expression at the mRNA and protein levels. Moreover, we have shown that linoleic acid (LA) reduces IL8 secretion. These results implicate the expression of CD14 molecules in human sebocytes and a competition of the proinflammatory activity of LPS through LA. Although CD14 expression was previously only determined in inflammatory cells, current reports have also postulated CD14 expression in keratinocytes. Therefore, we investigated the CD14 expression in SZ95 sebocytes by FACS analysis and by fluorospectrometry after labeling the cells with a fluorescent conjugated CD14 antibody. HL-60 promyelocytes served as control. While CD14 was detected on the surface and into the cytoplasm of HL-60 cells, SZ95 sebocytes only expressed CD14 into their cytoplasm. CD14 expression was enhanced in HL-60 cells by phorbol myristate acetate (PMA, 2.7-fold), but not by LPS and LA. LPS, PMA and LA did not alter CD14 expression in SZ95 sebocytes, but LPS and PMA markedly reduced the intracellular expression of CD14, a finding that indicates traslocation of CD14 to the cell surface. Interestingly, both LPS and PMA enhanced IL8 expression in SZ95 sebocytes, while LA (10^{-5} M) was unable to inhibit the stimulatory LPS effect. IL1α expression in SZ95 sebocytes was barely affected by LPS, PMA or LA. PMA, but not LPS and LA, stimulated total sebaceous lipids. In conclusion, LPS and PMA are likely to serve as proinflammatory signals in human sebocytes, respectively, inducing CD14-dependent and independent IL8 expression. The latter may be mediated by the PMA enhanced total sebaceous lipids. LA was unable to antagonize the proinflammatory activity of LPS.

230

Ageing Influences the Function of Human Epidermal Langerhans' Cells

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One clinical consequence of the ageing process is a reduced prevalence of allergic contact dermatitis in the elderly. Epidermal Langerhans' cells (LC) are key determinants of the skin's immune response to contact allergens. Following exposure to allergen subsequent migration of LC from the epidermis to draining lymph nodes is reliant upon receipt of two cytokine signals: one provided by tumour necrosis factor-α (TNF-α) and the other by interleukin (IL)-1β. We have investigated the effect of ageing on human epidermal LC numbers and their migration in response to exogenous TNF-α and IL-1β. Ten elderly (mean age 76 years) and 10 young (mean age 23 years) volunteers received intradermal injections of 200 U human recombinant TNF-α diluted in sterile saline and control injections of sterile saline at paired sites on photoprotected buttock. In a second cohort of volunteers 6 elderly (mean age 75 years) and 6 young (mean age 31 years) subjects received intradermal injections of 100 U IL-1β or sterile saline at paired sites on photoprotected buttock. Each injection site was subsequently biopsied under local anaesthesia either 2 h (TNF-α cohort) or 4 h later (IL-1β cohort). The frequency of $CD1a^+$ LC mm^{-2} of epidermis was assessed following direct immunoflourescence staining of epidermal sheets. The average baseline (saline treated) values for epidermal LC frequency in elderly subjects (852.6 ± 42.9) was significantly ($p < 0.01$) reduced as compared with young subjects (1126.7 ± 38.7). The response to TNF-α was significantly ($p < 0.05$) impaired in the elderly. Thus, the average percent reduction in LC frequency following injection was 9% as compared with 23% in the young. In contrast the response to exogenous IL-1β was retained in elderly skin with a 22% reduction in epidermal LC frequency as compared with a 19% reduction in young volunteers. These experiments indicate that in aged skin there is a reduction in frequency of epidermal LC coupled with an impaired migratory response to TNF-α. However, the response to IL-1β is retained with age. We conclude that aged skin is characterised by a decline in both LC frequency and function perhaps associated with a relative local deficiency of IL-1β. These factors may partly explain the reduced prevalence of allergic contact dermatitis in the elderly.

231

Reduced Susceptibility of Human Immature Dendritic Cells (IDC) to NK Cell Lysis after Interaction with the Opportunistic Yeast Malassezia Furfur (MF)

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The yeast *Mf*, normally present in the human skin microflora, is nevertheless a factor contributing to the severity of the chronic inflammatory skin disease atopic dermatitis (AD). The aim of this study was to assess whether coculture of *Mf* and human iDCs would affect subsequent interactions of DCs with autologous NK cells. iDCs preincubated with *Mf* showed a reduced susceptibility to NK cell killing when compared to untreated iDCs. Moreover, also when iDCs were treated with *Mf*-iDC coculture supernatant, a reduced susceptible to killing was observed, indicative of the presence of soluble factors. The numbers of CD40, CD80, CD83 and CD86+ DCs increased and HLA-DR expression was up-regulated after supernatant treatment. NK ($CD56^+/CD3^-$) cells were found in the dermis of skin biopsy specimens from healthy individuals and AD patients. However, in the AD patients $CD56^+$ cells were also found in the epidermis. These data suggest that NK cells and iDCs can interact, that *Mf* affects this interaction and that NK cells may play a role in regulating DCs. Furthermore, the data imply that DCs and NK cells can meet in the skin.

232

UVB Irradiation of Human Epidermal and Monocyte-Derived Langerhans Cells Perturbs the Antigen-Presenting Function: *In Vitro* Evaluation

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Exposure of the human skin to UV radiation causes an imbalance of its homeostasis and a reduced cutaneous immunity. The immunosuppression brought about by UVB is assumed to result in particular from the direct effects of UVB on the antigen-presenting function of the Langerhans cells (LCs), adhesion and costimulatory molecules being of crucial importance for the interaction of antigen-presenting cells with immune cells, especially T lymphocytes. The aim of the present study was to determine the differential effects of UVB on Langerhans cells antigen-presenting function by means of two models: (1) a human skin explant model irradiated with increasing levels of UVB (312 nm; 0.5–1 J per cm^2): after irradiation, LCs freshly isolated were tested for their allostimulating capacity in mixed lympho-epidermal culture (MLEC). UVB inhibited the lymphocyte proliferating response which was evaluated in the presence of ^3H thymidine: 0.75 J per cm^2 caused a 43% inhibition of the response; (2) a human monocyte-derived Langerhans cells (MoLCs) model: monocytes were cultured for 6 days in supplemented RPMI-1640, containing 50 ng per mL IL4, 100 ng per mL GM-CSF, 4 ng per mL TGFβ. *In vitro* UVB irradiation of MoLCs to increasing doses (0.005–0.03 J per cm^2) perturbed their allostimulating capacity in MLEC (0.03 J per cm^2 caused a 34% inhibition of the response) and modulated their functional expression of HLA-DR, CD54, CD86: MoLCs were stained for four-color analysis with the corresponding different monoclonal antibodies and analyzed on a double laser equipped FACSCalibur flow cytometer. Better knowledge of mechanisms involved in UV-induced immune suppression is essential for developing new strategies aimed at photoprotection and cancer prevention. Our results can be used and expanded to quantify UV-mediated Langerhans cell damage and the degree of immune photoprotection provided by various agents or sunscreens.

233

Naïve Human Langerhans Cells Show Phosphotyrosine Activity Without Stimulus

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The signalling pathways in naïve human epidermal Langerhans cells are poorly understood. Studies on murine Langerhans cells have shown that following stimulation with contact sensitizers, increased tyrosine phosphorylation can be detected. A similar result was observed following the investigation of human MHC classII positive cells following stimulation with haptens. However, in these studies Langerhans cells were cultured as an epidermal cell population and keratinocyte contamination was not taken into account. This may be important as keratinocytes can be induced to express MHC classII and could have been responsible for the effect observed. To investigate the intracellular signalling mechanisms involved in human Langerhans cells, we have studied phosphotyrosine activity in freshly isolated naïve Langerhans cells. The techniques used include an *in vitro* kinase assay using a phospho-specific antibody, in conjunction with Western blot analysis of these naïve cells. We observed the selective phosphorylation of two protein bands with molecular weights between 60 and 70 kDa. Preliminary results indicate that the protein bands belong to the src family of protein kinases, as shown using anti phospho-src antibodies. Keratinocyte contamination was evaluated with decreasing cell number and was found to negligible; enriched Langerhans cells were consistently 98% pure. These studies suggest that naïve Langerhans cells show phosphotyrosine activity and provide the basis for future work into the signalling pathways of these cells following their activation.

234

Induction of Dendritic Cell Maturation by Different Stimuli

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Human epidermal Langerhans cells (LC) aquire their antigens in the dermal tissue and migrate to the regional lymph node for presentation of the processed antigens to T-cells. During this migration the LC mature by changing their surface molecules and up-regulating, i.e. CD80, CD86 and CD83. Culture of CD34+ hematopoietic precursor cells in the presence of GM-CSF, TNF-α, SCF, FLT-3 L and TGF-β gives rise to immature dendritic cells (DC). A significant amount of these cells can be characterized as LC by the expression of Lag, intermediate levels of MHC II and low levels of CD86. We exposed these cells to different maturation stimuli and observed pronounced differences in phenotype and functional status. After exposure to LPS, poly I/C, TNF-α or a mix of TNF-α, IL1 and IL-6 a rapid up-regulation of maturation markers such as CD83, CD86, CD80 and MHCII appeared within two hours. Treatment of the cells with poly I/C or LPS led to a further increase of these markers until up to 6–12 h after stimulation, CD83 expression was back to prestimulation levels after 24 h. By contrast, monocyte-derived dendritic cells (MoDC) (generated in the presence of GM-CSF and IL-4) matured under poly I/C maintained their mature phenotype for at least 72 h. Treatment of immature CD34-derived DC with cytokines elevated the level of maturation markers for one to two days, then there was a slow decline. 30 min after TNF-α. Treatment: biosynthesis of MHCII molecules was stimulated as judged by immunoprecipitation. The *in vitro* generated LC were potent inducers of an allogeneic MLR, their potency increased after maturation. In conclusion, different maturation agents stimulate CD34-derived LC with different kinetics, suggesting different working mechanisms and possibly different functional implications. This finding is in contrast to observations on conventional monocyte-derived dendritic cells.

235

Immunophenotype and Apoptosis of Epidermal Dendritic Cells in Atopic Dermatitis Lesions Treated with Tacrolimus Oinment

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The newer immunomodulatory macrolactam ointments are new therapeutic options in atopic dermatitis (AD). Lymphocytes are well known target cells, but there is also evidence for an effect on mast cells, endothelial cells and dendritic cells (DC). Beside Langerhans cells (LC), a second epidermal dendritic cell population has been described in AD lesions, which is characterised by a high CD11b and FceRI expression but does not contain Birbek granules – the inflammatory dendritic epidermal cells (IDEC). Since previous studies with tacrolimus (FK506) ointment had shown a depletion of IDEC from the epidermis, the effects of FK506 and hydrocortisone on the immunophenotype and apoptosis rate of LC and IDEC were investigated *in vitro* and *in vivo*. *In vitro*, time kinetic studies with epidermal cell suspensions were performed in AnnexinV and TUNEL technique. A significant spontaneous rate of LC apoptosis was seen following LC isolation from normal human skin. Cell culture with different concentrations of FK506 did not influence this spontaneous apoptosis rate. All LC constantly expressed low amounts of CD95/Fas molecules. *Ex vivo* studies were performed in 15 AD patients treated double blinded with FK506 vs. hydrocortisone ointment. Epidermal dendritic cell phenotyping was performed from cell suspensions prepared from AD lesions before and after 1 week of therapy. Epidermal DC numbers – known to be increased in AD lesions – decreased during treatment by both FK506 and hydrocortisone. Thereby, only a slight decrease of LC was seen, in contrast to a 75% reduction in the cell number of IDEC. Apoptotic DC could be detected in all of our epidermal cell suspensions in variable percentages, including LC and IDEC. The rate of early apoptotic DC *in situ* was increased in hydrocortisone treated AD lesions for both LC and IDEC, whereas FK506 treatment did not lead to a higher percentage of apoptotic epidermal DC. An increased CD32 expression, but decreased FceRI, CD36 and CD80 expression was found on IDEC during FK506 treatment, witnessing the altered cutaneous microenvironment. In conclusion, therapeutic concentrations of FK506 do not seem to induce apoptosis in epidermal DC, but change the immunophenotype of epidermal DC during topical treatment of AD.

236

Correlation Between Th2 like PBMC Response and Atopy Patch Test Reaction to *M. Furfur* in Atopic Dermatitis Patients

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The yeast *Malassezia furfur* belongs to the normal cutaneous flora but is also considered to be one of the triggering allergens that can contribute to atopic dermatitis (AD). To illuminate the impact of circulating allergen specific T cells in AD, we have correlated the PBMC response with the *in vivo* skin prick test (SPT) and atopy patch test (APT) reactivity to *M. furfur* in AD patients ($n = 40$) and healthy controls (HC). None of the HC ($n = 16$) showed a positive *in vivo* reaction. The AD patients, of whom 18 had serum-IgE reactivity to *M. furfur*, could be subdivided into: SPT+/APT+ ($n = 12$), SPT+/APT– ($n = 12$), and SPT–/APT– ($n = 16$). The SPT+/APT+ and the SPT+/APT– groups but not the STP–/APT– group had a significant higher PBMC stimulation index (SI) ($p < 0.01$) than the HC. Interestingly, the SI values in the SPT+/APT+ group were significantly higher ($p < 0.05$) than in the SPT+/APT– group. In the *M. furfur* SPT+ AD patients ($n = 24$) a correlation between SI and the *M. furfur* APT reactions ($r_s = 0.62$, $p < 0.01$), but not between SI and *M. furfur* specific serum-IgE levels was found. Furthermore, production of the Th2 related cytokines interleukin(IL)-4, IL-5 and IL-13 ($r_s = 0.80$, $p < 0.001$; $r_s = 0.58$, $p < 0.01$; $r_s = 0.81$, $p < 0.001$, respectively), but not the Th1 related interferon-γ correlated with SI to *M. furfur* extract. Our data strongly suggest a relationship between circulating specific T cells, with Th2 like cytokine profile, and positive APT reactions.

237

Transcriptional Regulation of the Melanoma Cell Adhesion Molecule Gene in T Cells

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Extravasation and tissue infiltration of leukocytes and metastatic tumor cells require the regulated expression and function of adhesive surface molecules. Originally, the melanoma cell adhesion molecule MCAM/MUC18/CD146 was defined as a marker of tumor progression and mestastasis formation in human melanoma. MCAM influences later stages of the metastatic process, namely extravasation and establishment of new foci of growth. Recently, MCAM expression on CD3+ T cells infiltrating delayed-type hypersensitivity lesions of the skin, on synovial fluid T cells of rheumatoid arthritis patients and on distinct T leukemia cells, suggesting that this molecule might be involved in the extravasation and/or homing of activated T cells. In this study, we show that the mechanisms for up-regulation of MCAM in melanoma and in activated T cells are very similar. Using a combination of CAT reporter assays and semiquantitative RT-PCR, we observed that cAMP significantly increases transcription of MCAM in Jurkat T cells and in leukemia cell lines such as HL60 or K562 to levels comparable to those observed in cAMP-treated SB2 melanoma cells. Interestingly, MCAM transcription was not initiated in cells treated with reagents known to stimulate T cells, like phytohemagglutinin (PHA), phorbol esters (PMA), αCD28, or calcium ionophore (CaI) used in various combinations. On the contrary, the cAMP-dependent activation of the MCAM promoter was impeded upon treatment with PMA or PHA. The pyrimidine-rich initiator sequence Inr that overlaps the MCAM RNA start site was essential for promoter function and was shown to mediate the response to the stimulatory signals. DNA mobility shift experiments showed that in both melanoma and T cells, factors belonging to the basic HLH transcription factor family was induced upon cAMP treatment, a phenomenon that could be completely reversed upon addition of PMA. Taken together, these results indicate that transcription of the MCAM gene is very similar in both melanoma and T cells, confirming the hypothesis that MCAM could play a similar role in the migration and extravasation of these two cell types.

238

Regulation of Chemokine Gene Expression by IFN-γand IL-4 in Human Dermal Fibroblasts and Epidermal Keratinocytes

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Chemokines are known to play important roles for chemotaxis and activation of inflammatory cells in a phase of acute or allergic inflammation, such as atopic dermatitis. We investigated the effects of interferon-γ (IFN-γ) and interleukin (IL)-4 on chemokine gene expression in cell culture systems of normal human dermal fibroblasts (NHDF) and epidermal keratinocytes (NHEK). NHDF and NHEK were stimulated by tumor necrosis factor-α (TNF-α) or interleukin-1α(IL-1α) in the presence of IFN-γ or IL-4. After incubation, total RNA was extracted and reverse transcribed to cDNA. Amount of specific cDNA for IL-8, eotaxin and RANTES was determined by real-time quantitative PCR. Gene expression of these chemokines was standardized with expression of an internal control, GAPDH gene. In NHDF, TNF-α and IL-1α stimulated the gene expression of IL-8, eotaxin and RANTES with a different manner. IL-8 expression reached maximum at 6 h after stimulation, and declined thereafter, whereas the expression of eotaxin and RANTES was increased until 48 h. Interestingly, IL-1α hardly induced RANTES gene compared with TNF-α. IFN-γ and IL-4 inhibited IL-8 expression. Moreover, it was confirmed by quantitative PCR that eotaxin gene expression was stimulated by IL-4, and inhibited by IFN-γ. In contrast, RANTES expression was stimulated by IFN-γ. In NHDK, the manner of chemokine induction and its modification with IFN-γ and IL-4 is more than a little different from those in NHDF. These results suggest that the expression of these chemokines is differentially regulated by Th1 and Th2 cytokines.

239

Vasoactive Intestinal Peptide Plays a Central Role in Cytokine Network Around Keratinocytes

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VIP has been suggested to play some roles in inflammatory dermatoses such as atopic dermatitis and psoriasis. The aim of this study is to clarify the precise mechanisms of how VIP is implicated in the pathogenesis of these disorders. We investigated the expression of VIP and its receptors in normal human epidermal keratinocytes (NHEK), and a human epidermal keratinocyte cell line DJM-1 using reverse transcription-polymerase chain reaction and Northern blotting. Type I VIP receptor (VIP1R) mRNA was expressed in NHEK and DJM-1 cells, and the latter also expressed type II receptor (VIP2R) in lesser amounts. VIP transcript was not found in any cells examined. VIP1R mRNA was up-regulated by Th1 (IFN-γ), Th2 cytokines (IL-4) and TNF-α, as well as VIP itself, suggesting the presence of autoregulatory loop. VIP increased cAMP production and cell proliferation of DJM-1 cells, and also induced the production of inflammatory cytokines such as IL-6, IL-8, and RANTES. The production of cAMP and cytokines was abrogated by a VIP1R selective antagonist, indicating that VIP1R mediates these effects. Overall, these results suggest that up-regulation of VIP receptors by cytokines from inflammatory cells in the dermis enhances the proliferation and cytokine production of keratinocyte in response to VIP from nerve endings. This cytokine network around keratinocytes may be involved in the pathogenesis of inflammatory dermatoses.

240

Human Eosinophil Recruitment by Intradermally Injected Eotaxin: A Lightmicroscopic Study

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Eotaxin as a CC-chemokine is a potent eosinophilic chemoattractant *in vitro*. There is, however, only little information about the *in vivo* activity of eotaxin in humans. We therefore investigated the effect of eotaxin in normal skin as compared with uninvolved skin of patients with inflammatory skin diseases associated with peripheral eosinophilia. 50 ng and 100 ng eotaxin, respectively, dissolved in 50 µl saline were injected intradermally (control: 50 µl saline). After designated intervals 4 mm punch biopsies were taken and specimens processed by H&E and Giemsa staining. Apart from a transient wheal formation no other inflammatory signs at injection sites were seen. Injection of saline in normal skin induced no inflammatory changes. Histologically, challenge with eotaxin resulted in a discrete to moderate superficial dermatitis with a small number of eosinophils in a dose-dependent manner. In contrast to normal skin tissue from patients with blood eosinophilia showed a moderate (50 ng eotaxin) to heavy (100 ng of eotaxin) superficial perivascular lymphohistiocytic infiltrate with neutrophils and additionally numerous (10 per slide) to significant eosinophil accumulation (> 20 per slide). Moreover, in some cases 100 ng eotaxin induced histologic signs of vasculitis. Skin from patients with saline control revealed a moderate infiltrate but with a reduced number of eosinophils. Based on these observations we conclude that in human being eotaxin has also the capability to induce eosinophil-recruitment by binding to CCR3.

241

CXCR4 Expression in the Resolution of T Cell Mediated Cutaneous Inflammation

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The resolution of acute cutaneous T cell mediated inflammation is dependent on the clearance of the expanded T cell population. This involves apoptosis of the T cells, but additionally emigration of leucocytes probably plays a significant role. Chemokines and their receptors recruit lymphocytes to sites of inflammation, and regulate the compartmentalisation of the cells between lymphoid and peripheral nonlymphoid tissues. We have investigated the expression of chemokines and chemokine receptors during the induction and resolution phases of cutaneous tuberculin purified protein derivative-induced delayed-type hypersensitivity (Mantoux reaction) in 20 healthy volunteers. This was undertaken using 4-colour flow cytometric analysis of skin suction blister fluid cells and PBMC in conjunction with immunohistochemistry on skin biopsies. The clinical peak of the response occurred at day 3; however, T cell numbers were maximal at day 7. Non-specific inflammation associated with blister induction on control skin showed reduced CXCR4 (6.7%) and CCR7 (27.83%) expression on CD4+ lympocytes compared to those in the peripheral blood (PB) (12.13% and 87.75%, respectively). In contrast during Mantoux reactions, CXCR4 expression was increased at day 3 (33.31%) and at day 7 (24.26%) compared to PB CD4+ lymphocytes (15.27% and 9.10%, respectively). By day 14, CXCR4 expression was reduced (16.65%), but was still elevated compared to PB (11.33%). CCR7 expression at day 3 was increased (54.68%) compared to controls, but was lower than on PB CD4 + lymphocytes (81.27%). Decreasing CCR7 expression was noted during the course of the response at day 7 (30.29%) and particularly in the resolution phase at day 14 (11.97%) associated with stable expression within the PB (89.76% and 90.39%, respectively). Decreasing CCR7 expression during an immune response may indicate an increasing proportion of terminally differentiated CD4+ lymphocytes, but additionally represents a population of cells unable to home to peripheral lymphatic vessels during the resolution phase. Increased expression of CXCR4 during an inflammatory response may be important in retaining activated antigen-specific T lymphocytes within the inflammatory focus, while down-regulation may be required in order for these cells to emigrate during resolution of inflammation.

242

***In Vitro* Testing with Keratinocytes for Contact Allergy**

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The aim of this study is to investigate whether secretion of interleukin-8 (IL-8) proteins by keratinocytes following *in vitro* exposure to a contact allergen can be used to detect contact allergy. Suction blisters were made on skin of allergic and anergic subjects to urushiol, the contact allergen of poison ivy. Keratinocyte cultures were prepared from suction blisters of nontreated skin. They were then exposed to the allergen *in vitro*. Controls were the allergen solvent. Variable allergen concentrations, allergen exposure times and cell culture times were used. At the end of each culture time, IL-8 proteins of the culture supernatants were analyzed by ELISA. The concentration of IL-8 in the supernatants proved to be a successful way to distinguish between subjects who claimed to be allergic and who patch tested positive with a nontoxic concentration of urushiol and subjects who claimed no contact reactivity despite multiple contacts with the plant and who tested negative. In the allergic subjects, a correlation was established between the dose of the allergen and the IL-8 protein concentration in the supernatants. *In vitro* testing of contact allergies in patients makes possible an objective assessment of their allergic status without risking active sensitizations. The results indicate that the method may be used as an alternative method to animal models for testing consumer products before their marketing, thus avoiding ethical problems and problems related to interpretation of tests because of biological differences between animals and humans.

243

Interleukin-8 in Blister Fluid of Allergic Patch Tests is Chemotactic for T-Lymphocytes and a Detector of Grade of Contact Allergy

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The purpose of this study is to investigate interleukin-8 (IL-8) as a chemokine for T-lymphocytes and as a marker of magnitude of contact allergy. Urushiol, the contact allergen of poison ivy, was applied to allergic and anergic volunteers. Suction blisters of urushiol exposed and nonexposed skin were produced 48 h later and the fluids were analyzed for chemotactic activity for T-lymphocytes using modified Boyden chambers. Anti-IL-8-antibodies were added to the fluids to investigate their effect on the chemotactic activity. IL-8 protein concentration of the fluids was determined by ELISA and its correlation with visible test responses was investigated. Significant chemotactic activity for T-lymphocytes was only present in blister fluid from urushiol exposed skin and only from the allergic subjects. The chemotactic activity could be neutralized with the anti-IL-8 antibodies. There was an excellent correlation between the IL-8 protein concentration in blister fluids and the clinical appearance of the inflammatory reactions at the skin test sites. The results support the hypothesis that IL-8 in blister fluid is both chemotactic for T-lymphocytes and is a sensitive marker of an inflammatory response. Determination of the concentration of IL-8 proteins in blister fluid of patch tests may be of future use for objective assessment of clinically important contact allergies.

244

The Tole of Histamine in DNFB Induced Contact Hypersensitivity

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The functions of mast cells and histamine in immediate hypersensitivity reactions are well known, and it is also known that histamine participates in the regulation of various immunological functions. The aim of this study was to investigate the role of histamine in the regulation of dinitrofluorobenzene (DNFB) induced contact hypersensitivity. The abdominal skin of histidine decarboxylase (HDC) knockout mice was sensitized and five days later the dorsal surface of the ears were challenged with DNFB in aceton/olive oil. Ear thickness was measured at different time points, the skin-draining regional lymph nodes (DLN) were excised 48 h following the final chemical treatment and the cells were prepared for phenotypic analysis by flow cytometry. Routine histology and immunhistochemistry were also performed on ear specimens. The expression of IL-2, IL-4 and IFN-γ genes were examined by RT-PCR in the ear samles. The DNFB induced increase of the ear thickness was significantly higher in HDC−/− mice compared to wild type mice. We observed a significant decrease in the percentage of CD4+ Th and CD8+ Tc cells, and a significant increase in the percentage of B220+ B cells in the DLN of HDC−/− mice in contrast to wild type mice. In the ear specimens the majority of the infiltrating cells were neutrophils at 24 and 48 h post challenge of both groups, but the number of neutrophils was higher in HDC−/− mice compared to wild type mice. We observed significantly more CD45+ leukocytes in HDC−/− mice in contrast to wild type mice in immunhistochemistry. The rate of CD3+ T lymphocytes was not increased in DNFB sensitized mice, in comparison with the control (aceton treated) mice. IL-2, IL-4 and IFN-γ mRNA expressions were unchanged both in wild type and in HDC−/− mice irrespective of the DNFB treatment. These results suggest that the immunregulatory effects of histamine play important role in DNFB induced contact hypersensitivity.

245

Cellular *In Vitro* Reactivity in Nickel-Allergy: Early Increase of IL-4 Production Preceeding Specific Proliferation and IFN-γProduction

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In Nickel contact allergy antigen-specific T-cell proliferation can be assessed *in vitro*. In addition to specific proliferation, we examined the kinetics of mediator production and secretion pattern for IL-4 and IFN-γ after specific and mitogen-induced T-cell activation *in vitro*. Peripheral blood mononuclear cells (PBMC) of 6 nickel-allergic patients (history and patch test positive) and 4 nonallergic individuals were cultured in the presence of either medium, PHA, $NiSO_4$ or tetanus toxoid (TT). IFN-γ and IL-4 were measured by ELISA after 1 h, 2 h, 4 h, 24 h, 72 h and 144 h. The mRNA expression for IFN-γ and IL-4 was assessed by RT-PCR at the same time points. Actual cytokine production was also examined by flow cytometry using a surface and intracytoplasmatic staining. As control PBMC from nonallergic donors or unstimulated cultures were used. Whereas IFN-γ production and specific proliferation to Nickel and TT both increased at later time points (between 72 h and 144 h), there was an early IL-4 production. The latter was detectable both at RNA and protein level within 24 h. The transient IL-4 peak may contribute to Th1-favouring conditions, since IL-12 production was subsequently seen at FACS analysis.

246

Trafficking of CLA^+ Nickel-Specific $CD8^+$ and $CD4^+$ Lymphocytes is Directed by CXCR3 and CCR4 Chemokine Receptors, Respectively

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Allergic contact dermatitis results from a T cell response to haptens applied to the skin, with $CD8^+$ T lymphocytes providing the most relevant mechanisms of tissue damage, and $CD4^+$ cells exerting both effector and regulatory functions. Thus, a differential recruitment of these two T cell subsets may have a direct impact either in the amplification or in the resolution of the immune reaction. Here, we investigate the migratory behavior of CLA^+, nickel (Ni)-specific T lymphocytes obtained from the peripheral blood of allergic individuals. *In vitro* migration assays indicated that IP-10 (CXCL10) and TARC (CCL17) represent the most potent chemoattractants for $CD8^+$ and $CD4^+$ T cells, respectively. MCP-1 (CCL2) induced a significant migration of both the subsets. Accordingly, CXCR3 (IP-10 receptor) was uniformly expressed on $CD8^+$ T lymphocytes independently from the cytokine profile, whereas Th1, but not Th2 $CD4^+$ lymphocytes expressed significant levels of CXCR3. Conversely, CCR4 (TARC receptor) was detected on the vast majority of Ni-specific $CD4^+$, but only on a limited percentage of $CD8^+$ cells. CCR2 (MCP-1 receptor) was uniformly expressed by both $CD4^+$ and $CD8^+$ subsets. RT-PCR from skin biopsies, showed that MCP-1 was already highly present at 16 h, whereas TARC and IP-10 reached a peak at 48 h after hapten challenge. Supernatants of IFN-γ-activated keratinocytes, which represent the most important source of IP-10 in the skin, induced comparable migration of $CD4^+$ and $CD8^+$ cells. However, neutralization of IP-10 with specific Ab determined a much higher inhibition of $CD8^+$ (80%) compared to $CD4^+$ (40%). Our data indicate that the axis CCR4/TARC, and CXCR3/IP-10 direct Ni-specific $CD4^+$ and $CD8^+$ skin recruitment, respectively.

247

Serum Eosinophil Cationic Protein (ECP), Myeloperoxidase (MPO), Tryptase, Eotaxin and Th-2-Like Cytokines in Dermatitis Herpetiformis

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Dermatitis herpetiformis (DH) is a subepidermal bullous disease characterized by a neutrophilic and eosinophilic infiltrate, together with activated lymphocytes. So far the role of soluble mediators such as cytokines and chemokines has not been studied in the peripheral blood of DH, as well as the serum levels of myeloperoxidase (MPO), eosinophil cationic protein (ECP) and tryptase. We investigate the presence of possible alterations in interleukin (IL)-4, IL-5, eotaxin, MPO, ECP and tryptase concentrations in 40 DH patients (19 females, 21 males, median age 22.5 years, range 5–80) in order to study the potential pathogenetic role of Th2-like cytokines and chemokines and to confirm the role of granulocytes and mast cells in determining typical lesions of DH. As controls, sera from 20 healthy volunteers (11 females, 9 males, median age 24 years, range 25–52) were collected and tested in parallel. IL-4 and IL-5 and eotaxin serum contents were determined by a solid phase enzyme-linked immunosorbent assay (ELISA). A double antibody radioimmunoassay was used for measuring the serum levels of MPO, ECP and tryptase. In DH patients, IL-5 serum levels were within the normal range (mean 1.53 pg per mL; values ranging from 0.0 to 6.6 pg per mL) except for a sole patient in which increased values were demonstrated (75.0 pg per mL). IL-4 levels were found in the normal range (mean 2.68 pg per mL; values ranging from 0.0 to 14.0) as well as eotaxin ones (mean 99.48 pg per mL; values ranging from 30.5 to 248.7 pg per mL) in all DH patients. IL-5, IL-4 and eotaxin levels were normal in the sera of healthy controls. MPO serum levels showed a significant increase (1173.74 ± 430.36 ng per mL); a moderate increase for ECP serum values was even documented (19.52 ± 14.16 ng per mL) and, interestingly, a significant correlation between the levels of MPO and ECP was detected ($t = 4.3$; $p < 0.001$). Finally our study did not document the presence of tryptase in the serum of DH patients examined. In healthy controls, MPO serum levels were within the normal range (180–326 ng per mL) while the mean ECP values was 7.9 ng per mL (range 2.3–16). Tryptase was not documented in the healthy group too. The detection of Th2-like cytokines, eotaxin and tryptase levels in serum of DH patients can not be considered a marker of disease activity while the increased serum levels of MPO and ECP we obtained confirm the utility of these mediators as serologic parameters of neutrophil and eosinophil activation.

248

The Chemokine Eotaxin is Elevated in the Serum of Patients with Dermatitis Herpetiformis Duhring

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Dermatitis herpetiformis (DH) Duhring is a papulovesicular eruption mainly involving shoulders, elbows and back. The disease is characterized by granular papillary IgA deposits of unclear pathophysiologic significance and a dermal infiltrate rich in neutrophils and eosinophils. These cells are implicated in the pathogenesis of the disease through secretion of tissue-destroying enzymes. Homing of these cells can be attributed to cytokines such as eotaxin, IL-13 and TNFα. To evaluate the serum cytokine milieu necessary for eosinophil maturation and chemotaxis, we analyzed serum samples of 8 patients with DH by ELISA for eotaxin, IL-5 and IL-13. Additionally, we examined lesional cryostat sections for eotaxin deposition and expression of the eotaxin receptor CCR3 on the infiltrating cells by immunohistochemistry. Serum analysis revealed an about 3-fold higher eotaxin concentration in sera of DH patients (median: 0.6 pg per mL, range 0.28–1.09) compared to healthy controls (median: 0.2 pg per mL, range 0.03–0.43). IL-5 and IL-13 serum levels as detected by ELISA were below the respective detection limits (32 pg per mL and 3 pg per mL). Immunohistochemistry of lesional cryostat sections demonstrated eotaxin staining in subepidermal papillae. The expression of the eotaxin receptor CCR3 could be shown on lesional inflammatory cells. Thus eotaxin is not only present in lesional skin but is also elevated in the serum of DH patients. Eotaxin exerts its chemotactic effect on eosinophils and TH2 cells by binding to CCR3 expressed on lesional infiltrating cells. Therefore eotaxin might contribute to the perpetuation of DH and may be suitable as a serum marker for the inflammatory activity of the disease.

249

Estimation of Soluble Receptors of Interleukin 2 and Tumor Necrosis Factor α Levels in Serum of Systemic Sclerosis Patients

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Pro-inflammatory cytokines are regarded to be involved in autoimmunologically determined fibrosis in the course of systemic sclerosis. Soluble interleukin 2 receptor (sIL-2R) and tumor necrosis factor alfa (sTNFαRI) as activation markers of lymphocytes are postulated to play some role in evaluation of disease activity. The aim of our study was to estimate whether serum levels of sIL-2R and sTNFαRI could be of help in systemic sclerosis (SSc) course monitoring. The study was performed in the group of 17 SSc patients (12 with limited – lSSc; and 5 with diffuse form – dSSc; 3 male and 14 female, aged 31–70 years). The control group comprised 19 healthy people (1 male and 18 female, aged 16–60 years). Degree of skin fibrosis was estimated on the basis of Total Skin Score (TSS) according to Kahaleh et al. Measurements of serum cytokines were performed three times (I, II, III) in one year follow-up period. Serum levels of sIL-2R and sTNFαRI were performed by ELISA method (Quantikine R&D Systems, INC, USA). The obtained results were analysed by statistical methods including Friedman test, Wilcoxon test for paired data and both Mann–Whitney and Kruksal-Wallis tests. It was demonstrated that levels of both soluble receptors were increased in serum of SSc patients (sIL-2R mean values in I – 2418.659 pg per mL; in II – 2142.612 pg per mL, in III – 1967.447 pg per mL; sTNFαRI mean values in I – 1402.659 pg per mL, in II – 1342.829 pg per mL, in III – 1292.929 pg per mL) in comparison to healthy people (mean sIL-2R – 1258.982 pg per mL and mean sTNFαRI – 938.491 pg per mL). The above correlations were statistically significant at $p < 0.05$. In one-year follow-up period, when treatment was introduced, clinical improvement was observed and serum levels of the soluble receptors decreased (not statistically significant difference $p > 0.05$). Measurements of serum sIL-2R and sTNFαRI levels could be of help in estimation of disease activity in SSc patients.

250

Induction of Interleukin-8 in Keratinocytes by Unique Pseudomonas Aeruginosa-Derived Pathogen-Associated Molecules

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Epithelial infections with the gram-negative bacterium *Pseudomonas aeruginosa* (PA) often show a massive influx of neutrophils resulting in pustule formation. The mechanism of this neutrophil accumulation is as yet speculative. Although bacteria-derived neutrophil attractants may play a role and complement activation is important, we speculated that epithelial Interleukin-8 (IL-8) induction could also contribute to this phenomenon. Therefore the aim of this study was to analyse whether PA is capable of stimulating IL-8-production in keratinocytes. We investigated different PA strains (ATCC strains) for induction of IL-8 mRNA-expression in primary keratinocytes as well as HaCat cells by RT-PCR and protein release using an in-house IL-8-ELISA. IL-8 mRNA-induction was found in keratinocytes that were in contact with both, mucoid and nonmucoid types of heat-inactivated PA. Further analyses revealed that IL-8-inducing activity can also be found in bacterial culture supernatants indicating that IL-8-inducing activity is released from bacteria. In order to investigate whether lipopolysaccharide is the IL-8-inducer we stimulated primary keratinocytes with PA supernatants in the presence of the LPS-inhibitor Polymyxin B. However, we did not find any influence of IL-8-induction. Biochemical characterization experiments demonstrated that one part of the IL-8-inducing activity can be extracted with organic solvents indicating that it is a lipid, whereas another part is most likely a protein. RP-HPLC of both the lipid-like and hydrophilic IL-8-inducing activities revealed elution in single fractions indicating separate Pathogen Associated Molecules (PAMs) that induce IL-8 synthesis. Focussing on the proteinaceous activity we further purified IL-8-inducing factors utilising additional chromatographic steps. Analysis of the fractions corresponding to this IL-8-inducing activity by SDS-PAGE reveals a single protein band. A further molecular characterization is currently in progress. In conclusion, our findings support the hypothesis that human skin keratinocytes recognise PAMs that are unique for highly pathogenic, biofilm-forming microorganisms such as *P. aeruginosa* bacteria to mount an epithelial defense reaction by inducing epithelial neutrophil attractants such as IL-8.

251

Elafin – A Serine-Protease Inhibitor with Antimicrobial Activity, Inducible by Supernatants of Pseudomonas Aeruginosa

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Elafin is a peptide like serine-protease inhibitor, secreted during differentiation of keratinocytes. It is believed to be important in the regulation of elastase-mediated tissue damage and might play an important role in maintaining the stratum corneum integrity of the skin. Recent studies provided evidences for the antimicrobial activity of elafin against *P. aeruginosa*. Therefore, we were interested, whether supernatants of *P. aeruginosa* are capable of inducing elafin expression in keratinocytes. In our study we could demonstrate using realtime-PCR that elafin mRNA-expression was up-regulated during differentiation of cultured keratinocytes. Supernatants of various *P. aeruginosa* strains stimulated elafin mRNA-expression and protein release, which we analysed by specific ELISA. The increase of induction was higher compared to stimulation with IL-1β and TNF-α in both, differentiated and nondifferentiated keratinocytes. In nondifferentiated cells the relative increase was much higher (100 fold) than in differentiated cells (6 fold), which though exhibited higher constitutive mRNA expression (500 fold). Differences in elafin transcripts did not correlate to secreted elafin by keratinocytes. After stimulation secreted elafin concentrations were similar in differentiated or nondifferentiated cells. This is explainable by the fact that elafin is bound covalently to extra-cellular components by transglutaminase, which is expressed in differentiated keratinocytes. In this study we could not confirm bactericide effect against *P. aeruginosa* as described previously but bacteriostatic properties as demonstrated for different strains in liquid cultures. We conclude that the role of elafin in human skin depends on the level of keratinocyte-differentiation. Constitutively expressed in differentiated cells it maintains the integrity of the stratum corneum. In basal keratinocytes it appears to be an inflammatory response factor with serine-protease inhibitory and antimicrobial activity.

252

Interleukine-4 Plasma Levels in Patients with Different Types of Psoriasis

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Interleukin 4 (IL-4) is a pleiotropic cytokine produced by T lymphocytes and mast cells. It exerts its action on many different cells including fibroblasts, endothelial cells, T and B lymphocytes and many others. Since mast cell–fibroblast interactions are postulated to be involved in psoriasis, the aim of our study was to evaluate IL-4 levels in plasma of psoriatic patients in comparison to healthy donors. The study comprised 30 patients with active psoriasis (erythrodermic form, psoriasis arthropatica and eruptive guttatae psoriasis) – Group 1, 15 patients with stable psoriasis – Group 2 and 10 healthy donors – Group 3. Severity of the disease was evaluated in each patients (PASI score). The patients were off any systemic treatment for at least 4 weeks and off local treatment, with the exception of emollients, for at least 2 weeks. IL-4 levels were evaluated before the treatment and 3 weeks after an in-patient intensive treatment (Ingram method or methotrexate regimen). IL-4 levels were measured by ELISA method (capture – purified mouse antihuman IL-4, standard – rhIL-4, antibody – biotin rat antihuman IL-4, all Phar Mingen; Labsystem Multiskan RC at 492 nm). Before the treatment, in Group 1, IL-4 levels ranged from 930.7 to 12.2 pg per mL (mean 224.6) and after the 4-week treatment it decreased and ranged from 315.0 to 4.7 pg per mL (mean 87.5) ($p < 0.05$). Before the treatment, in the Group 2, IL-4 levels ranged from 29.1 to 0.0 pg per mL (mean 8.4) and after the treatment ranged from 15.6 to 0.0 pg per mL (mean 3.8) ($p < 0.05$). Whereas in the control group (Group 3) IL-4 levels ranged from 84.2 to 0.0 pg per mL (mean 9.95). The changes in IL-4 levels were pronounced in the group with active, extensive psoriasis which well responded to treatment. When exacerbation of the disease or resistance to the introduced treatment was noted during the hospitalisation period, IL-4 levels showed a tendency to stay at the same level. The obtained results suggest that IL-4 is the next cytokine, out of the whole network already, to be involved in the course of psoriasis.

253

Histochemical Analysis of Macrophage Migration Inhibitory Factor (MIF) in Psoriasis Vulgaris

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Psoriasis is a persistent cutaneous disease characterized by skin inflammation with infiltration of immunocytes, such as lymphocytes and monocytes/macrophages concomitant with abnormal epidermal hyperproliferation. We previously showed that serum macrophage migration inhibitory factor (MIF) level and its production by peripheral blood mononuclear cells (PBMCs) of the patients with psoriasis closely correlated with the severity of clinical symptoms; however, the precise role of MIF in psoriasis remains to be clarified. The current study was carried out in order to elucidate the possible involvement of MIF in the pathomechanism of psoriasis, using immunohistochemistry and *in situ* hybridization. Contrasting to elevated serum MIF in psoriasis, MIF-positive staining in lesional psoriatic epidermis was significantly decreased examined by immunohistochemical analysis using an anti-MIF antibody. The intensities of positively stained MIF bands of psoriatic epidermis were significantly lower than those of normal controls by Western blot analysis. Consistent with these findings, we found that MIF mRNA concomitantly decreased in the psoriatic lesions using *in situ* hybridization. Taken together theses results, it is considered that the major source of elevated serum MIF in psoriasis could be PBMCs, and that different MIF levels in psoriatic lesions and circulation may reflect the pathology of the chronic inflammatory skin disease with regard to epidermal hyperproliferation and systemic inflammation.

254

Dimethylfumarate Inhibits TNF-Induced CD62E Expression in a NF-κB-Dependent Manner

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Fumaric acid esters (FAE) are thought to improve psoriasis by altering leukocyte, keratinocyte and/or endothelial functions. To determine specificity, kinetics and molecular mechanisms of different FAE in their ability to inhibit endothelial cell activation, we analyzed CD62E and CD54 expression in endothelial cells *in vivo* and *in vitro*. In lesional skin of psoriatic patients, oral FAE treatment resulted in a marked reduction of CD62E but not of CD54 expression on dermal microvessels. Using human umbilical vein endothelial cells (HUVEC), dimethylfumarate (DMF) almost completely inhibited TNF-induced CD62E, but not CD54 expression at concentrations $\leq 70\,\mu M$, mimicking the situation *in vivo*. A 60-min DMF preincubation was sufficient to block TNF-induced CD62E expression for up to 24 h. In contrast, equimolar concentrations of methylhydrogen fumarate (MHF), the hydrolysis product of DMF, did not suppress TNF-induced CD62E expression. Likewise, all FAE other than DMF were ineffective. Using CD62E, NF-κB or AP-1-responsive promoter constructs, DMF inhibited TNF-induced activation of the CD62E and the NF-κB but not of the AP-1 promoter construct. In summary, at a dose range $\leq 70\,\mu M$, DMF appeared to be a specific inhibitor of CD62E expression in a NF-κB-dependent manner.

255

Dimethylfumarate Inhibits Tumor Necrosis Factor-Induced Nuclear Entry of NF-κB/p65 in Human Endothelial Cells

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Dimethylfumarate (DMF), a diester of fumaric acid, is known to inhibit TNF-induced activation of endothelial cells *in vivo* and *in vitro*. To characterize the molecular basis of DMF action, we analyzed TNF- and/or VEGF-induced tissue factor expression in human endothelial cells in culture. We show that DMF inhibited TNF-induced tissue factor mRNA transcription and protein expression, as well as TNF-induced DNA binding of NF-κB in endothelial cells. In contrast, DMF has no effect on VEGF-induced tissue factor protein and mRNA expression as well as DNA binding of EGR-1 and constitutive SP1. Employing immunoblots of nuclear extracts and FACS analysis of nuclei isolated from green fluorescent protein-NF-κB/p65-transfected endothelial cells we show that DMF inhibited the TNF-induced nuclear entry of p65. This is not due to inhibition of TNF-signaling down to IκB, because DMF does not alter TNF-induced IκBα phosphorylation and IκBα degradation. Also the constitutive shuttling of inactive p65/IêB complexes into and out of the nucleus is not altered by DMF. We conclude that DMF inhibits NF-κB-induced gene transcription at the level of the nuclear entry of p65, after its release from IκB.

256

Evaluation of the Convenience of the Cd7 Marker in the Identification of Sézary Cells

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Cutaneous T-cell lymphomas (CTCL) are characterized by the infiltration of the skin by T lymphocytes of the CD2+ CD3+ CD4+ CD45RO + DR + phenotype. CTCL represent a unique model in tumoral terms because both tumoral and reactive cells are T lymphocytes. Till now no specifical marker of tumoral cells has been described in the literature. Nevertheless, since the idiotype of the TCR represents a unique marker for a malignant clone of T lymphocyte, the clonal origin of tumoral cells allows us to think that the TCR-Vβ clone corresponds to the tumoral cell. Concurrently, loss of expression of CD7 is considered as a diagnostical criterium for CTCL because of its frequent publication and would be specific of Sezary cells. But this hypothesis remains unconfirmed. Our goal is to determine whether this CD7- population represents the Sézary cells. Our study turns on one patient, cytologically confirmed and exhibiting more than 50% circulating Sezary cells. A sorting, by cell sorter, has been realised in order to harvest the CD4+ CD7+ and CD4+ CD7- populations, which were then submitted to a cytological evaluation on the basis of the cerebriform nucleus of Sézary cells. We found for this first patient that both CD4+ CD7+ and CD4+ CD7- populations contained Sézary cells, so this finding suggests that CD7 is not an appropriate marker to identify Sézary cells. Moreover we were able to note that this cerebriform appearance was lost after a few days of *in vitro* culture with IL-2, IL-7 or IL-15, therefore this aspect could be the consequence of the sensitivity of the lymphocytes to the cutaneous microenvironment.

257

***In Vitro* Expression of Interleukin-15 and its Receptor by Normal Human Keratinocytes and *In Vivo* Expression in Cutaneous T-Cell Lymphomas Mycosis Fungoides and Sézary Syndrome**

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Interleukin (IL)-15 exerts similar biological activities as IL-2 through common receptor transducing subunits, IL-2/IL-15Rβ and γc chains. The specificity of the receptor for IL-2 or IL-15 is obtained after association with a third chain lacking transducing activity (IL-2Rα, IL-15Rα). Unlike IL-2, IL-15 mRNA are ubiquitously expressed but their translation is tightly controlled and IL-15 protein is seldom produced under physiological conditions. The aim of this work was to study IL-15 and IL-15 receptor expression firstly by normal human keratinocytes *in vitro* and secondly *in vivo* in cutaneous T-cell lymphomas (mycosis fungoides and Sézary syndrome), psoriasis and atopic dermatitis. We observed that unstimulated normal human keratinocytes expressed IL-15 and IL-15 receptor mRNA and also IL-15 and IL-15Rα proteins *in vitro*. Their expression was increased after stimulation of cells by IFNα or γ. Interestingly, it appeared that IL-15Rα protein was mainly located in the nucleus, whereas IL-15 protein was detected in the cytoplasm and/or at cell membrane. *In vivo*, we did not detect IL-15 and IL-15Rα protein expression both in normal human skin and cutaneous lesions of atopic dermatitis and psoriasis. However, IL-15 and IL-15Rα protein expression was observed in keratinocytes for IL-15 and in T-cell infiltrates for IL-15Rα of cutaneous T-cell lymphoma, strongly suggesting a possible role of IL-15 in this disease. The stimulus responsible for IL-15 induction in cutaneous T-cell lymphoma remains however, to be identified.

258

High Immunomodulatory Potency of Consensus Interferon Alfacon-1 on Melanoma Cell Lines

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The interferon(IFN)s are known for their antiproliferative, antiviral and immunoregulatory effects *in vitro* and *in vivo*, though these effects vary in magnitude depending on the cell type targeted. In regard to melanoma cells, IFN-β and IFN-γ have been shown to exert stronger antiproliferative and immunomodulatory potencies than IFN-α. In the present study we investigated the effects of the synthetic type I consensus IFN alfacon-1 compared to IFN-α2b, IFN-β and IFN-γ in 5 melanoma cell lines. The cell surface expression of HLA class-I/–II molecules and of the apoptosis-associated proteins FasR/CD95, bcl-2 and bcl-xS was determined by flow cytometry. The sensitivity towards FasR/CD95-mediated apoptosis was measured using fluorescence double-staining with annexin-V and propidiumiodide. We found a strong dose-dependent up-regulation of HLA class-I on all 5 cell lines by IFN-β, IFN-γ and alfacon-1, instead of a slight increase by IFN-α. IFN-β and alfacon-1 significantly revealed antiproliferative action in 4 of 5 cell lines, with highest potencies by IFN-β. IFN-α and IFN-γ showed antiproliferative effects in 1 of 5 and 2 of 5 cell lines, respectively. IFN-β, IFN-γ and alfacon-1 significantly sensitized 4 of 5 cell lines towards FasR/CD95-mediated apoptosis, whereas IFN-α showed no response. In conclusion, our data indicate the consensus IFN alfacon-1 as a potent immunomodulatory agent in malignant melanoma *in vitro*, suggesting further studies on the *in vivo* use of this new type of IFN.

259

Fibroblasts Derived from Chronic Diabetic Ulcers Differ in their Response to Growth Factors Compared to Controls

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Patients with diabetes mellitus experience impaired wound healing, often resulting in chronic foot ulcers. Healing can be accelerated by application of growth factors like platelet-derived growth factor (PDGF). We investigated the mitogenic responses, measured by ^{3}[H] Thymidine incorporation, of fibroblasts cultured from diabetic ulcers, non diabetic ulcers, and non lesional diabetic and age matched controls to recombinant human PDGF-AB, epidermal growth factor (EGF), fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I). We determined the optimal concentration of these factors, and investigated which single factor, or combination of factors, added simultaneously or sequently, induced the highest mitogenic response. For single growth factor additions: (1) diabetic ulcer fibroblasts needed more EGF to reach their maximum mitogenic value compared to non lesional controls (p#0.04) and (2) PDGF-AB induced the highest mitogenic response in all four categories of fibroblasts, this was a significant finding for diabetic ulcer fibroblasts when compared to IGF-I (p#0.007) and bFGF (p#0.05) and (3) the optimum stimulation achieved by IGF-I was significantly lower for diabetic ulcer fibroblasts vs. controls (p#0.003). Simultaneous addition of combinations of growth factors to diabetic ulcer fibroblasts always produced a higher stimulatory response than sequential additions (significant for PDGF-IGF, FGF-PDGF and FGF-PDGF; $p < 0.01$). The highest response was obtained by the simultaneous addition of PDGF-AB and IGF-I (synergistic effect, $p < 0.05$ vs. all other combinations), this was not observed in the chronic ulcer and age matched controls. In conclusion, the combination of PDGF-AB and IGF-I seems more promising than PDGF-AB alone for clinical application in chronic diabetic wounds.

260

Expression of SMAD 7 in Cultured Fibroblasts From Patients with Keloid Scarring

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The SMAD Proteins have been recently described as the intracellular signalling pathway of the transforming growth factor β (TGFβ). SMAD's consist of three groups: receptor regulated SMAD's (SMAD 1, 2, 3, 5, 8), common SMAD (SMAD 4) and the inhibitory SMAD's (SMAD 6 and 7). Receptor–ligand interaction is leading to phosphorylation of the receptor regulated SMAD's. Inhibitory SMAD's terminate the TGFβ signal in a negative feed-back loop. TGFβ plays an important role in the pathogenesis of keloid scarring. Keloids develop after skin injury and tend to grow invasively into the surrounding healthy skin. TGFβ has been reported to be responsible for the ongoing, massive over-expression of extracellular matrix components in keloids. Until today it remains unknown why the TGFβ signal in keloids is not terminated. Therefore we investigated the expression of the inhibitory SMAD 7 in cultures of fibroblasts from keloid tissue biopsies. For comparison fibroblast cultures from patients not suffering from keloids were established. Semi-confluent fibroblast cultures were stimulated with 20 ng TGFβ-1 for 30 min, 1 h, 2 h, 6 and 24 h. After stimulation mRNA was isolated for real-time RT-PCR. In addition fibroblasts were lysed after stimulation to investigate protein expression by western-blotting. First results showed that mRNA expression of SMAD 7 after stimulation with TGFβ-1 is reduced in keloid fibroblasts compared to fibroblasts of normal skin. Expression of SMAD 7 protein in keloid fibroblasts investigated by western-blotting showed a diminished amount of SMAD 7 protein compared to normal skin. These results show that the expression of SMAD 7, the major inhibitory SMAD protein, is reduced on protein and on mRNA level in cultures of keloid fibroblasts. The decreased SMAD 7 concentration in keloid fibroblasts may shift the balance between activating SMAD's and inhibitory SMAD's in favour of the activating side leading to an ongoing cellular proliferation.

261

Keratinocytes Downregulate Expression of Connective Tissue Growth Factor in Fibroblasts

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The role of keratinocytes for regulation of fibrogenic cytokines in fibroblasts was investigated. Fibroblasts were cultured in multiwell plates together with keratinocytes in semipermeable cell culture inserts. By this method, the two cell types were physically separated but could exchange diffusible factors. In presence of keratinocytes, expression of CTGF mRNA in fibroblasts was suppressed by half 6 h after establishment of cocultures, and inhibition persisted throughout a 48-h culture period. Keratinocytes suppressed both serum or TGF-β_1 stimulated CTGF mRNA in fibroblasts and the effect was also seen with conditioned medium from keratinocytes. Basal or TGF-β_1 stimulated CTGF protein expression was also suppressed by at least half in the presence of keratinocytes. TGF-β_1 mRNA in fibroblasts was less affected by coculturing with keratinocytes and corresponding protein unchanged. Furthermore, keratinocytes suppressed collagen I expression at the level of mRNA and protein synthesis while general protein synthesis was unaffected in our model. TNF-α is a possible candidate for the suppressive effects on CTGF and collagen I mRNA but was not operative in our model. Taken together, the results provide a mechanism that may explain, at least partly, the clinical observation that epithelial coverage is important for down-regulation of connective tissue activity during the end-stage of wound healing.

262

Functional Activation of Calcineurin by Differentiation Promoting Agents in Human Epidermal Keratinocytes and Inhibition by Cyclosporin A

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Systemic cyclosporin A (CsA) is an effective treatment for psoriasis but its mechanism of action is incompletely understood. IL-2 production in T cells depends on activation of the phosphatase calcineurin and translocation of the transcription factor NFAT accompanied by calcineurin to the nucleus. CsA blocks T cell activation by inhibiting this pathway. Reverse transcriptase/PCR and western analysis demonstrated the presence of calcineurin A2 and B in human epidermal keratinocytes. Cultured human keratinocytes were treated with differentiation- promoting agents that also induce a rise in intracellular calcium, fixed, stained sequentially with monoclonal antibodies to calcineurin A and calcineurin B subunits, Cells were visualized by immunofluorescence and confocal microscopy and the proportion of cells showing nuclear positivity counted. Calcineurin A and calcineurin B colocalized and appeared predominantly cytoplasmic in untreated keratinocytes. TPA (50 nM), TPA plus ionomycin (1 μM), and increased extracellular calcium (1.5 mM $[Ca^{2+}]_o$) induced translocation of calcineurin A and calcineurin B subunits to the nucleus by 2 h that was maximal at 4 h ($n = 3$, $p < 0.0001$ compared to control) and sustained to 18 h in response to TPA and TPA plus ionomycin. Pre-treatment of keratinocytes with CsA (1 μM) reduced the mean percentage (±SD) of cells showing nuclear positivity for calcineurin A from 94 ± 1 to 10 ± 2.3 in response to 1.5 mM $[Ca^{2+}]_o$ for 4 h and from 65 ± 4 to 10 ± 5 in response to TPA for 4 h ($n = 3$, $p < 0.0001$). All three agents also induced translocation of calcineurin A and B to the plasma membrane at 4 h that was inhibited by CsA. These data provide evidence that differentiation-promoting agents activate calcineurin in human keratinocytes resulting in nuclear translocation and that CsA inhibits this signaling pathway.

263

Cyclosporin A Directs Cyclophilin B to the Constitutive Secretory Pathway in Human Keratinocytes

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Cyclosporin A (CsA) is effective treatment for inflammatory skin diseases. The mechanism of action of CsA in T-cells is well characterised, however, its effects in skin are less well understood. Cyclophilin B (CypB) is a 21-kDa protein capable of both binding CsA, and catalysing the isomerisation of certain peptide bonds. In T-cells, CypB is secreted in response to treatment with CsA, and is thought to contribute to the effects of CsA. As yet, the physiological role of CypB remains unclear. The aim of this study was to determine if CypB is expressed in skin, and if CsA modulates CypB in cultured keratinocytes. CypB was shown to be expressed by human keratinocytes using RT-PCR and immunoblotting. Human keratinocytes were treated with CsA (1 μM) for up to 96 h then either (1) fixed and double labeled with anti-CypB antibody and a probe for the golgi apparatus or (2) culture medium was collected (either immediately or up to 96 h after removal of CsA from cells) and analysed by immunoblotting. In untreated cells, CypB was found in both the nucleus and cytoplasm, but after treatment with CsA, CypB colocalised with the golgi apparatus and the plasma membrane. CypB accumulated in the medium of treated keratinocytes in a time dependant manner, and continued to be secreted upon removal of CsA from the culture medium. Secretion of CypB could be inhibited by pretreatment of cells with brefeldin A, an inhibitor of constitutive secretion. These data show that CsA promotes secretion of CypB by keratinocytes and suggest that CypB may play a role in mediating the action of CsA in inflammatory skin disease.

264

Vascular Endothelial Growth Factor-C is Involved in Lymph Node Localization of Human Melanoma Metastases

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Melanoma can metastasize through different routes comprising direct invasion of surrounding tissue and spread via the vascular and/or the lymphatic system. However, little is known about the molecular mechanisms which guide metastasis spreading and localization in distinct tissues. Among the cytokines of the vascular endothelial growth factor family (VEGFs), VEGF-C and VEGF-D have been shown to be involved in tumor-induced lymphangiogenesis and lymphatic metastasis formation. These cytokines are ligands for VEGFR-3/Flt-4, a tyrosine kinase receptor almost exclusively localized to lymphatic vessels in the adult. To characterize the role of VEGF-C and VEGF-D in human melanoma, we have analyzed expression of these cytokines in cultured human melanoma cell lines derived from cutaneous and lymph node metastases. VEGF-C mRNA and protein were preferentially expressed in lymph node-derived tumor cell lines. On the other hand, VEGF-D was detected at extremely low level in all the melanoma cell lines examined. Immunohistochemistry on melanoma specimens from which the cell lines were derived demonstrated a correspondence in VEGF-C expression between cultured cell lines and tumor samples. In addition, immunohistochemical analysis of 46 human melanoma specimens confirmed the preferential expression of VEGF-C in lymph node (16/21) vs. cutaneous (6/25) metastases. Our findings indicate that VEGF-C may be involved in melanoma metastasis spreading via the lymphatics and open new prospect for therapy.

265

Intracellular Signalling of Phytoestrogens in Relation to VEGF Receptors Expression by Hair Follicle Cells

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Many studies indicate the impact of phytoestrogens (PHO) in normal physiology of humans and animals. They have demonstrated to possess numerous biological properties such as inhibition of tyrosine kinase activity. The mechanisms by which these PHO exert their effect are multifactorial and tissue-specific. The impact of PHO on hair growth was less extensively investigated. Published reports indicate that treatment of keratinocytes with selective PKC inhibitors stimulates their proliferation and *in vivo* stimulates murine hair growth (Yokoo et al. 1999). These data led us to study firstly the effect of PHO on PKC and then after to test the hypothesis that the vascular responses of hair follicle cells *in vitro* are linked to the effects of PHO on PKC activity and expression. We evaluated by Western- blot analysis the effect of two total plant extracts: soya and pueraria in comparison with two free forms (genistein, daidzein) on the expression of PKC α and δ isoforms. We also evaluated their effect on PKC activity in cultured human hair follicle dermal papilla cells (DPC). In parallel, we evaluated by Western-blot and RT-PCR the effect of PHO on VEGF receptors (flt-1 and Flk-1) expression on DPC. The direct effect of PHO on hair growth was assessed on human hair follicles. Our results showed a dose-dependent inhibition of PKC α and δ expression in the presence of PHO and specific inhibitors of PKC isoforms. PKC activity was also seen to be markedly decreased in the presence of genistein, daidzein and soya extracts (42%inhibition for 1 μM genistein and 44% for soya extract at 1 μg per mL). The PKC activity was less reduced in the presence of pueraria extract at 1 μg per mL (10% inhibition). The inhibition induced by genistein is similar to that of a PKC inhibitor: Calphostin C (45%inhibition). However, we observed an increase of VEGF receptors expression in the presence of PHO. Specific inhibitors of PKC also promoted Flt-1 expression by DPC. Our results showed that Flt-1 and KDR expression within hair DPC is mainly mediated through the PKC pathway. This signalization is modified in the presence of PHO and chemical protein kinase inhibitors: an overexpression of VEGF receptors is observed in DPC. It will be of interest to examine the functional impact and significance of protein kinase C modulation on the established vasculature of the hair follicle.

266

Disappearance of the Capillary Network in Alopecia Areata is due to Growth Inhibition of Human Microendothelial Cells by Pro-Apoptotic Molecules Released from Peripheral Mononuclear Blood Cells (PBMC)

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Alopecia areata (AA) is considered to be a T-cell mediated immunologic disease with genetic predisposition, however, its etiopathogenesis has not yet been fully elucidated. Histopathologic examination in the early inflammatory phase of AA shows a strong peribulbar infiltrate with disappearance of the vascular network surrounding the hair follicle. The aim of the present study is to investigate signaling molecules previously identified as key mediators in alopecia areata as well as peripheral mononuclear blood cells (PMBCs) for their significance in regulating endothelial cell growth. Cultured human microendothelial cells (HMECs) were treated with different signaling molecules as well as activated and nonactivated PBMCs of both AA patients and nonaffected individuals. After 3 h incubation HMECs were analyzed for their apoptotic behavior using a photometric enzyme-immunoassay (cell death detection ELISA). Necrosis was excluded by measuring lactate dehydrogenase activity released into cell culture supernatants. IL-1α, IL-1β, IL-2 (all 10 ng per mL), TNF-α (20 ng per mL) and TGF-β (4 ng per mL) induced apoptosis in HMECs without necrosis. In contrast, IL-4 (100 ng per mL), IFN-γ (0.5 ng per mL), IL-8, IL-10, and IGF-1 (all 10 ng per mL) did not lead to apoptosis or necrosis in HMECs. Furthermore, supernatants of Phythamagglutinin (PHA) stimulated PBMCs induced significant apoptosis without necrosis in a 1 : 8 dilution exclusively in AA patients but not in nonaffected individuals. However, using a lower concentration (1 : 1) of supernatants from PMBCs apoptosis and also necrosis were detected in both supernatants from AA and in nonaffected individuals. We suggest that proinflammatory cytokines like IL-1α,-1β and TNF-α as well as proapoptotic molecules released from PMBCs are responsible in AA for the disappearance of the capillary network surrounding the hair follicle, subsequently leading to initiation of the catagen phase.

267

Sphingosine Kinase is Expressed in the Human Immortalized Keratinocyte Cell Line HaCaT

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Formation and maintenance of the normal epidermis is a balanced tissue formation where regulation of proliferation and differentation of keratinocytes is highly important. Pathological reactions of the skin like atrophy or hyperkeratosis may be considered as directly related to proliferation and programmed cell death, i.e. apoptosis. Sphingosine-1-phosphate (SPP) is a highly bioactive sphingolipid metabolite exerting both intracellular and extracellular actions in mammalian cells. Various stimuli, serum and platelet-derived growth factor (PDGF), activate sphingosine kinase, the enzyme that phosphorylates sphingosine yielding SPP. Intracellular SPP was shown to influence signaling pathways leading to cellular proliferation and inhibition of apoptosis in various cell types. On the other side, SPP exerts extracellular functions as the ligand for the G-protein-coupled receptor EDG-1, which influences cell migration. Previous studies showed that ceramides are mediators of apoptosis induced via tumor necrosis factor α (TNF α). As the intracellular balance of SPP and ceramides may determine the fate of the cell, we investigated the role of SPP in the keratinocyte cell line HaCaT. In the present study, we demonstrate for the first time the expression of sphingosine kinase in the keratinocyte cell line HaCaT. Sphingosine kinase experiments using cytosol of HaCaT showed a time and concentration-dependent formation of SPP. In addition, SPP-formation could be blocked using competetive inhibitors of sphingosine kinase. Our data indicate that sphingosine kinase may be involved in cellular processes concerning keratinocyte proliferation and differentiation.

268

Augmentation of Perilipin and PPARau Expression is Associated with the Accumulation of Intracellular Lipids in Hamster Sebocytes *In Vitro*

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Sebaceous glands are important skin appendages and sebum excretion is considered to be associated with the functional maintenance of the cutaneous surface as a biological barrier. Sebocytic differentiation sequentially occurs with accumulating cytoplasmic lipids. On the other hand, it is well known that adipocytes are differentiated to accumulate abundant lipids in the cytoplasm. Perilipin has been located at the periphery of the lipid droplets and associated with lipid metabolism in adipocytes. In addition, PPAR participates in lipogenesis in the differentiated cells. However, it remains unclear whether perilipin and PPAR participate in the accumulation of intracellular lipids in sebocytes. Therefore, we have investigated the expression of perilipin and PPAR in differentiated hamster sebocytes by Western blot and immunohistochemical analysis. When hamster sebocytes were treated with insulin, 5ad-dihydrotestosterone (5ad-DHT) and interleukin 6 (IL-6), the expression of perilipin was augmented along with increase in the accumulation of intracellular lipids. Furthermore, the expression of PPARac was augmented by these factors but that of PPARgw was negligible. On the other hand, insulin augmented the expression of perilipin and the lipogenesis in primary hamster adipocytes whereas 5ar-DHT and IL-6 did not influence them. In addition, neither PPARaD nor PPARgn expression was modulated in these factors-treated hamster adipocytes. Therefore, these results suggest that the augmentation of perilipin and PPARae expression may be associated with lipogenesis in differentiated hamster sebocytes, and further that the regulation of perilipin and PPARs expression in sebocytes may differ from that in adipocytes.

269

Assessment of the Skin Metabolism of Two Stable Antioxidant Precursors to a Free Tocopherol, Tocopheryl-Glucoside vs. Tocopherol Acetate, in an Epidermal Equivalent

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Among the enzymes involved in the epidermal barrier function, two, an esterase and a β-glucocerebrosidase can serve to release bioactive compounds. The aim of this study was to assessed the skin penetration and metabolism of two stable antioxidant precursors, the α-tocopherol acetate and the δ-tocopheryl-glucoside. The study was performed with a reconstructed human epidermis (RHE). After an isotopic dilution of the tritiated molecules, 0.1% of the precursor solubilise in a cosmetic oil have been deposited both on infinite and finite dose. Radio-HPLC analysis has been used to determine the amount of precursors which have penetrate and to quantify the conversion into free tocopherol at 18 H for infinite dose study, and at 2, 6 and 18 H for finite dose study. After an exposure of 18 h radio-HPLC analysis show penetration of the two precursors in the RHE. In infinite dose, about $18 \pm 7\%$ and $47 \pm 13\%$ ($n = 9$) of the total δ-tocopheryl-glucoside was converted into δ-tocopherol in the stratum corneum (1 tape stripping) and in the epidermis, respectively. No conversion of α-tocopherol acetate to α-tocopherol was found in the RHE. Accordingly, only the kinetic of conversion the δ-tocopheryl-glucoside has been studied. The results show that 45, 76 and 89% ($n = 4$ for each time) were converted into free tocopherol at 2, 6 and 18 h, respectively. These findings show that epidermal equivalent can be used to assess the metabolism of topicaly applied molecules. No free α-tocopherol was converted from α-tocopherol acetate. On the other hand the gluco-conjugated tocopherol is a valuable precursor to deliver into the skin free tocopherol, firstly the glucoconjugation increases the thermal and photochemical stability of δ-tocopherol and secondly the reservoir effect coupled with the enzymatic conversion constitutes an efficient controlled released of free tocopherol *in situ*.

270

Barrier Function of the Axillary Stratum Corneum

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Axillary stratum corneum has a unique environment, being subjected to hydration stress as a result of sweat and its enclosed anatomical location. To determine whether this region contains phenotypically altered axillary skin we have investigated the stratum corneum barrier function. Both *trans*-epidermal water loss (TEWL) measurements, in a carefully controlled setting, and corneosurfametry (CSM) revealed that the axilla had a reduced barrier function compared to the forearm. HPTLC analysis of stratum corneum lipids demonstrated signficantly elevated levels of fatty acids, ceramide and particularly cholesterol in the axilla compared with forearm. Attenuated total reflectance Fourier transformed infrared (ATR FTIR) spectrometry was used to investigate lipid lamellae organisation. Preliminary data indicated that the organisation of the axillary barrier lipids differed from that of the forearm, which may explain the observed barrier effects. To investigate whether a specially formulated aerosol antiperspirant (AP) product, containing a moisturising cream, could be used to supplement barrier function, Skin Surface Water Loss (SSWL) analysis was performed on AP treated skin. AP treatment reduced by approximately 50% the water loss from the site compared to untreated control. By contrast, repeating the treatment with the AP in the absence of the moisturising cream gave a similar water loss to that of the untreated control. Conclusion: (1) axillary stratum corneum has a deficient barrier; (2) the levels of axillary stratum corneum lipids are significantly elevated compared to forearm: (3) perturbation of the stratum corneum lipid ratios may explain barrier deficiency; (4) a specially formulated aerosol AP product can significantly improve the water holding capacity of the stratum corneum, and promote normal barrier function.

271

Visual Scaliness of Human Skin Correlates to Decreased Ceramide Levels and Decreased Stratum Corneum Protease Activity

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Variations in scaliness of the human skin with age or in conditions of barrier perturbation have often been attributed to alterations in stratum corneum hydration as well as a disturbed lipid content. The protein compartment of the epidermal barrier has thereby received relatively little attention. The stratum corneum proteases SCCE (chymotryptic enzyme) and SCTE (tryptic enzyme) are believed to play a major role in the regulation of the desquamation process of the stratum corneum. We have previously shown that barrier disruption by tape stripping leads to a temporary reduction in the activity of these proteases, suggesting that reducing the degradation rate of intercellular cohesive structures may contribute to the initial phase of barrier recovery. In the present study we investigated whether variations in scaliness of the skin on different anatomic sites (lower leg, the inner forearm and the face) would be associated with changes in protease activity and ceramide content in the upper stratum corneum. After normalization to total protein a significant and inverse correlation was observed between skin scaliness and protease activity as well as ceramide level. This effect was most pronounced for the phytosphingosine based ceramide subfraction. The data suggest that both a chronical decrease in active proteases and a diminished activity, caused by a decreased amount of water retaining ceramides, may contribute to the induction of a scaly dry skin.

272

Slow Internal Release of Bioactive Compounds Under the Effect of Skin Enzymes

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Among the enzymes involved in the maintenance of the epidermal barrier function (1), two of them, a β-glucocerebrosidase and an esterase can be exploited as a route for the delivery of bioactive compounds. These two enzymatic activities are first evidenced and assayed in a normal skin, using appropriate substrates. A more detailed analysis of the requierements for hydrolysis by the first enzyme was obtained after cloning and overexpressing it. Hydrolysis occurs as long as the leaving group is good enough ($pk < 10$) and there is no steric hindrance at the glycosidic bond being broken. This strategy is firstly illustrated with an antioxidant δ-tocopherol owing to the general importance of that class of compounds. The corresponding glucoconjugate is hydrolysed with release of δ-tocopherol the antioxidant properties of which are higher than those of the α-derivative. Moreover this strategy of slow release of the compound prevents a pro oxidant effect. Release of any compound can obtained by introducing a spacer between the glucose unit and the molecule to be released. This spacer may also provide an extra bioactivity. This situation is examplified with the release of retinoic acid, using two different spacers, hydroquinone (depigmentent) and glycerol (control agent of water loss) the two spacers allowing the control of the rate of the release. It should be noticed, in relationship with practical applications, that glucoconjugation increases the thermal and photochemical stabilities of δ-tocopherol; it was checked that for the δ-toco-conjugate, the delivery of the compound is actually observed *in vivo*, at the stratum corneum level, and this with a reservoir effect. This strategy can be extended to diverse situations: care and therapy of the skin, delivery of compounds which presently are internalized in the body from patches and drugs which have shown a hepatic toxicity by oral route.

273

Development of New 5-α Reductase Inhibitors

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5-α reductase, the enzymes system that metabolizes testosterone into dihydrotestosterone (DHT), occurs in two isoforms, the type A which represents the ''cutaneous type'' and the type 2 which is located mainly in the epididymis, seminale vesicles, prostate,…Androgen-dependent skin disorders, such as seborrhoea, acne, female hirsutism and/or androgenetic alopecia are among the most common diseases encountered by the dermatologist. Thus the development of new compounds that effectively inhibit the type 1 isoenzyme of 5-α reductase are of major importance for dermatological purpose. The aim of this work is to present new compounds which are able to inhibit significantly 5-α reductase type 1. The efficiency of these molecules was tested on human skin fibroblasts in culture. Cells were cultured with radiolabelled testosterone and the evaluation of DHT formation by thin-layer chromatography has been applied to evaluate 5-α reductase activity. Using this protocol, we have demonstrated that butyl avocadate, methyl ricinoleate and Pygeum Africanum were effective 5-α reductase type 1 inhibitors.

274

Cyclosporine a Reduces Retinoic Acid Degradation and Increases The Expression of a Retinoid-Regulated Gene in Cultured Keratinocytes

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Cyclosporine A (CsA) and synthetic retinoids is often used to treat severe psoriasis. Both endogenous retinoids (e.g. retinoic acid; RA) and CsA are metabolized by cytochrome p450-dependent enzymes. The aim of this work was to study if cultured keratinocytes exposed to CsA exhibit altered RA metabolism and signaling. We exposed keratinocytes to 10 μM CsA or liarozole, a retinoic acid metabolism blocking agent, and studied the metabolism of [^{3}H]retinol and [^{3}H]RA in the cells by radiochromatography. In parallell experiments we measured the mRNA expression of a retinoid-regulated gene, CRABPII, by real-time quantitative PCR. It was found that CsA altered the metabolism of both [^{3}H]retinol and [^{3}H]RA. In the former case, a 10–20-fold increase of cellular [^{3}H]retinol and [^{3}H]RA, and a 10-fold decrease of 3,4-[^{3}H]didehydroretinol and 3,4-[^{3}H]dide-hydroretinoic acid was found. In the latter case the cellular content of [^{3}H]RA was increased 3̃-fold compared to control cells. Liarozole also increased cellular [^{3}H]RA, but the effect was less pronounced than with CsA in both cases. Furthermore, in cells exposed to CsA the mRNA expression of CRABPII increased about 2–3 times, peaking at 8–16 h. A novel mechanism is proposed by which CsA affects psoriasis: through increased endogenous retinoid-signaling, mimicing the effect of cytochrome p450-inhibitors, e.g. liarozole.

275

Cellular Uptake, Metabolism and Biological Effectiveness of 13-*cis* Retinoic Acid on SZ95 Sebocytes are Regulated by Extracellular Binding Protein Levels

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13-*cis* Retinoic acid is rapidly absorbed into human sebocytes, is selectively isomerized to high levels of all-*trans* retinoic acid and exerts an antiproliferative effect by binding the retinoic acid receptors in normal culture conditions *in vitro* (J Invest Dermatol 115: 321–7, 2000). In this study, we provide evidence that the levels of extracellular binding protein plays an important role for cellular uptake, metabolism and biological effectiveness of 13-*cis* retinoic acid on human sebocytes. The addition of highly concentrated bovine serum albumin (20-fold), that selectively binds nonaromatic retinoids, to the serum-free medium resulted in a decreased and controlled uptake of 13-*cis* retinoic acid into SZ95 sebocytes; almost constant levels of 13-*cis* retinoic acid for 72 h were detected after a single treatment. In addition, delayed isomerization of 13-*cis* retinoic acid into all-*trans* retinoic acid was observed. The intracellular concentration of all-*trans* retinoic acid after 13-*cis* retinoic acid treatment was nearly similar to that of 13-*cis* retinoic acid at 24 h, however, it was 2- to 8-fold higher at 48–120 h after treatment. In a parallel experiment, the antiproliferative effect of 13-*cis* retinoic acid (10^{-7} M) was diminished by adding high levels of bovine serum albumin in the serum-free medium compared to the effect detected in experiments with normal levels of bovine serum albumin. These results indicate a critical function of serum albumin, as extracellular retinoid-binding protein, in reducing the intracellular concentration of active retinoids and in diminishing the biological effect of retinoic acids on human sebocytes.

276

Addition of Pentoxifylline Reduces the Side-Effects of Fumaric Acid Esters in Treatment of Psoriasis

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The efficacy of fumaric acid esters (FAE) for treatment of psoriasis is well proven, however, patients often discontinue the FAE-treatment due to gastrointestinal side-effects and flush, which are supposed to be mediated by TNF-α release from monocytes. We speculated that a reduction of FAE-induced TNF-α secretion should improve the tolerability of the compound. Therefore, we compared FAE therapy and the combination of FAE plus pentoxifylline (PTX), a potent suppresser of TNF-a release. 44 patients with moderate to severe psoriasis vulgaris were included in this open-label prospective trial. In group A (FAE monotherapy), 16 out of 21 patients showed adverse effects, 4 patients ceased the FAE treatment. The incidence was slightly lower in group B (combination therapy FAE plus PTX) where 12 patients out of 23 reported adverse effects leading to withdrawal from the study in 4 cases. Each of the adverse effects, were less frequently reported in the patients receiving PTX coapplication. Concerning the severity of all side-effects a significant reduction was found in the patients with combination therapy. Our data supports the hypothesis that the FAE-associated side-effects are TNF-α mediated and indicates that adding PTX seems to increase the tolerability of this therapy regime.

277

Comparative *In Vivo* Pharmacokinetic Study of 8-Methoxypsoralen (8-MOP) Penetration into Human Skin by Microdialysis after Creme, Bath or Oral Delivery

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Photochemotherapy with oral or topical psoralens combined with long wave ultraviolet light (PUVA) is a standard treatment regimen for various dermatological disorders. However, until now clinical treatment schedules are based largely on phototoxicity testing and determination of 8-MOP serum kinetics, while 8-MOP tissue kinetics are still missing. Providing concentration-time profiles with a high temporal resolution microdialysis has been shown to be a suitable method for the characterisation of transdermal drug transport *in vivo*. We used the microdialysis technique to compare tissue concentrations in humans after topical (bath and cream) and oral administration of 8-MOP. The microdialysis is based on a diffusion process through a semipermeable membrane located at the tip of a microdialysis catheter. The catheter is constantly perfused with a physiologic solution (flow rate 0.5–3 μL per min). Substances that enter the catheter through diffusion are transported to the outlet tube and can be analysed in the dialysate. Because proteins cannot pass the membrane, it is pharmacodynamically relevant unbound fraction of a substance that can be determined by microdialysis. 8-MOP was applied topical and systemical in 8 healthy volunteers in a cross over study. Dialysate sampling was performed up to 6 h in 20 min intervals. Plasma samples were taken up to 10 h after 8-MOP application. Plasma and tissue concentrations of 8-MOP were determined by LC with double masspectography. Plasma concentrations after oral 8-MOP administration were significantly higher than after topical 8-MOP application while tissue concentrations were significantly lower after oral 8-MOP and showed a large variance. Tissue concentrations after oral 8-MOP reached a maximum after 1–3 h. There was no significant difference between bath PUVA and cream PUVA. Tissue concentrations after 8-MOP bath and 8-MOP cream decreased rapidly within 30 min. We conclude that cream PUVA can be a substitute for bath PUVA therapy, as the pharmacokinetic profiles of bath and cream PUVA are very similar. Our data further indicate that topical 8-MOP application is superior to the oral route as skin concentrations are higher and more predictable.

278

Anti-Angiogenic Properties of Temozolomide

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Temozolomide (TMZ) is a methylating agent used in the therapy of malignant melanoma. It is well established that certain chemotherapeutic agents display potent antiangiogenic properties which may be part of their antitumor activity. We sought to determine whether TMZ is capable of inhibiting angiogenesis, which would favour its use in antiangiogenic tumor therapy regimens. We examined the inhibitory activity of TMZ on angiogenesis in the chicken chorion-allantois membrane (CAM) assay, in the matrigel assay and a methylene blue- based proliferation assay, using human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC). In the CAM assay angiogenesis was induced by bFGF or conditioned medium from NIH3T3 fibroblasts. The vessel count in TMZ treated CAMs was significantly lower than in the positive control. In the matrigel assay, the alignment of HUVEC and HDMEC to form capillary-like structures was significantly inhibited by TMZ as evidenced by the count of ''capillary intersections''. Likewise, in the proliferation assay, TMZ showed an inhibitory action which corresponded to the well known inhibitory potential of vinblastin, which was used in comparison. The concentration of TMZ in all three assays (5 μg per mL) corresponded to serum levels obtained after cyclic high dose regimens (200 mg per m^2 body surface). The antitumor activity of TMZ may therefore in part be due to its antiangiogenic properties. Further studies will have to show whether lower concentrations of TMZ retain their antiangiogenic properties, thus enabeling the design of orally available antiangiogenic treatment schedules of malignant melanoma.

279

Pharmacological Study of Levocetirizine in IgE-Dependent Hypersensitivity Cutaneous Reaction in Grass Pollen Allergic Volunteers: Demonstration of Mediator Release and Eosinophil Recruitment Modulation by Levocetirizine

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This study aimed to quantitatively determine the effects of oral administration of levocetirizine (5 mg per day during 6 days) on the release of mediators (soluble VCAM-1, LTC_4, TNFa histamine, tryptase), the vasopermeability and the cell recruitment induced by antigenic stimulation in 15 grass pollen allergic subjects during a 25-h- *in vivo* IgE-mediated hypersensitivity cutaneous reaction. This was a monocentre, randomized, double-blind, placebo controlled, cross-over study in 15 subjects allergic to grass pollen (mean age = 27.1 years). The methodology was based on the skin chamber technique: at the 4th day of oral treatment, skin blisters were created on the forearm and skin chambers were applied on the dermis. At the morning of the 5th day, pollen vs. control medium were introduced into skin chambers and renewed hourly during 6 h, left until the 24th hour and collected at the 25th hour. Rebuck's glasscoverslips were then applied during one hour to establish cell recruitment during the late phase reaction. For each mediator the AUC of the difference between control and pollen chamber were used to compare levocetirizine and placebo. A modulatory effect of levocetirizine as compared with placebo was observed on the activation of the vascular endothelium: – the VCAM-1 level as well as the protein level in the first 6 h of pollen-induced reaction were significantly inhibited under levocetirizine (VCAM-1 mean AUC = 2.3 ng per h per mL per cm^2 and protein mean AUC = 4.3 mg per h per mL per cm^2) compared with placebo (VCAM-1 mean AUC = 10.3 ng per h per mL per cm^2 and protein mean AUC = 7.3 mg per h per mL per cm^2), [respectively, $p = 0.011$ and $p < 0.02$]. There were no major effects of levocetirizine on the other measured mediators: histamine, tryptase, TNFα and LTC_4. A decrease in the recruitment of eosinophils was observed under levocetirizine for the 4 subjects with high migration (eosinophils cell greater than 6%): median eosinophil counts were 11.6% under placebo and only 1.8% under levocetirizine. Nevertheless, no conclusion can be drawn for subjects presenting a low eosinophilic migration. These results require further investigation in order to confirm this major modulatory property of levocetirizine on eosinophils. In conclusion, our results shows 3 main inhibitory effects of levocetirizine on VCAM-1 release, vascular permeability and eosinophil recruitment in response to allergic stimulation.

280

Arsenic Trioxide Induces Apoptosis of Sezary Cells *In Vitro*: Evidence for a Partially Caspase-Independent Pathway and Potentiation by Ascorbic Acid (Vitamin C)

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Arsenic trioxide (As2O3) has recently been shown to exhibit antiproliferative and apoptogenic properties against malignant cells of either myeloid or lymphoid lineage. In this study, we investigated the effects of As2O3 on the proliferation and the viability of the Sezary cell line HUT-78 and peripheral blood mononuclear cells (PBMCs) derived from patients with Sézary syndrome (SS) showing a high proportion of tumoral cells in the peripheral blood. Viability/apoptosis were assessed by using flow cytometric analysis after propidium iodide and annexin-V staining, Terminal dUTP Nick End Labelling (TUNEL), cell cycle analysis, mitochondrial transmembrane potential ($\Delta\psi m$) alterations, and detection of processed caspase 3 by Western blot and flow cytometry. The results show that a As2O3 10 μM is needed to induce significant cell death of HUT-78, whereas PBMCs from patients with SS exhibit a much greater sensitivity to apoptosis induction, in a time- and dose-dependent manner. Thus, 50% of cells were annexin positive after 72 h incubation with As2O3 2 μM. Both kinetics and quantitation of As2O3-induced apoptosis of PBMCs from patients with SS were found in the same range than those of PBMCs from healthy blood donors. Double staining with anti-TCRBV/anti-CD3 monoclonal antibodies showed that the proportion of tumoral cells remained stable after As2O3 treatment, suggesting a similar sensitivity of Sézary cells and normal lymphocytes from a given patient to As2O3-induced apoptosis. Ascorbic acid 100 μM was shown to potentiate As2O3-induced Sezary cell death at a greater extent than PBMCs from normal individuals, while interferon α showed no synergistic effect. Caspase 3 activity was shown to be induced by treatment of tumoral cells with As2O3 by Western blot and flow cytometry. Nevertheless, As2O3-induced apoptosis of Sézary cells was not altered by specific caspase inhibitors (ac-DEVD-CHO, ac-LEHD-CHO) and only partially inhibited by the pan-caspase inhibitor Z-VAD-fmk. These results demonstrate that As2O3 synergizes with vitamin C to induce Sezary cell death *in vitro* at clinically achievable concentrations, through a pathway partially independent of caspase activation. These data warrant clinical studies in order to evaluate *in vivo* the therapeutic efficacy and the toxicity of arsenic trioxyde in patients affected with cutaneous T-cell lymphoma

281

Evaluation of the Avene Spring Water Effect on Oxygen Radicals Generation

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During cellular metabolism, cutaneous inflammatory process or pathogenesis of human diseases, oxygen radicals can be generated. The release of reactive oxygen species (ROS) is indicative of cell stress and their overproduction induces an important cellular alteration especially of the plasmic membrane components, of the proteins and of the genomic material. Avene spring water (ASW) has been widely used in the treatment of various inflammatory skin diseases (atopic dermatitis and psoriasis). In order to evaluate the ASW effect (from 0 to 100% in millipore water) on oxygen radicals generation, we have used different *in vivo* and *in vitro* models. We have first analysed the ASW effect on ROS production by phorbol ester (TPA)-stimulated mouse peritoneal macrophages, using a chemiluminescence assay. The effect of ASW on macrophage oxidative metabolism was investigated by measuring the peak of luminol-enhanced chemiluminescence response. The chemiluminescence inhibition (ROS production inhibition) following ASW treatment was significant with 50% and 75% of ASW. We have secondly analysed the *in vitro* ASW potential protective role against alteration of the main cellular targets (membrane lipids, ADN and proteins) induced by ROS generated during ultraviolet (UVA) irradiation. So, we used UVA-irradiated human skin cells in which we measured the lipid peroxidation level by assaying the TBARS (ThioBarbituric Acid Reactive Substances), the DNA damages using the comet assay, and the oxidized proteins by immunodetection of carbonyl group. The membrane lipid oxidation assay (TBARS) clearly showed the protective effect of ASW which is maximal for 50% and 75% ASW. The qualitative and quantitative evaluation of DNA strand breaks showed the genomic protection efficiency of ASW with a maximal effect at 50% of ASW. Moreover, the ASW also decreased the protein oxidation in a dose-dependent manner. These studies clearly show a significant antioxidant activity of the ASW. In conclusion, by its antiradical properties, Avene spring water preserves the cutaneous tissue from dramatical effect of free radicals during inflammation process of the skin.

282

Tocopherol Precursor Evaluation: Effect on Oxygen Radicals Generation in Cutaneous Cells

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The skin is always in contact with oxygen. In fact, endogenous and exogenous oxygen radicals could be generated during cellular metabolism, prooxidant stress from environment or during cutaneous pathogenesis of human diseases. The release of reactive oxygen species (ROS) is indicative of cell stress and their overproduction induces an important cellular alteration especially of the plasmic membrane components, proteins and nucleic acids. In order to evaluate the antioxidant activity of delta tocopheryl glucopyranoside, an α tocopherol precursor, we used *in vitro* models. Oxidative stress is investigated by treating cutaneous cells with chemicals agents, like H_2O_2 or with physiological proinflammatory molecule, the TNFα (Tumor Necrosis Factor). Firstable, we used the fluorescent probe, 2′-7′-dichlorofluorescin-diacetate (H_2DCF-DA) to assess ROS generation in the intracellular partition, constituting a precocious step of the oxidative stress. In this approach, the δ tocopheryl glucopyranoside strongly reduces the intracellular ROS. Then, we analysed the δ tocopheryl glucopyranoside potential protective role against alteration of cellular targets (membrane lipids and genomic DNA) induced by ROS. So, we measured the lipid peroxidation level by assaying the TBARS and also the DNA damages using the comet assay. Starting from the lowest concentration (5 μg per mL), δ tocopheryl glucopyranoside efficiently protect DNA and membrane from oxidative attack. In comparison with reference molecules (α tocopherol), these studies clearly show a significant antiradical activity of the δ tocopheryl glucopyranoside. Like this, by its antioxidant properties, topical application of δ tocopheryl glucopyranoside could constitute a preventive factor of skin aging by supporting physiological mechanisms to maintain or restore a healthy skin barrier.

283

UV-Induced Suppression of Chronic CHS Leads to *In Vivo* Formation of Suppressor T Cells that are Capable of Immunosuppression of Acute as well as Chronic CHS

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Chronic CHS is induced by repeated application of hapten, resulting in site-directed shift to the immediate onset of CHS, peaked usually at 1–3 h postchallenge. We could currently show that, in addition to immunosuppression in conventional acute CHS induced by sensitization followed by challenge only once, ultraviolet (UV) light is also capable of suppression of this type of CHS, when animals were exposed to low doses of UVB before challenge. However, it still remains unclear if the suppression of chronic CHS by UV is mediated by *in vivo* generation of suppressor cells. To address this issue, C3H/HeJ mice were first UV-exposed or left untreated, were sensitized with dinitrofluorobenzene (DNFB). 5 days later, mice were challenged with the right earlobes, followed by repeated DNFB application at every third day, totally 12 times. Subsequently, the immediate ear swelling response was observed in non-UV-exposed mice upon final challenge, which was significantly prevented with UV exposure before sensitization, indicating that UV suppresses chronic CHS. Interestingly, suppression of chronic CHS is only seen in C3H/HeJ mice, known to be UV resistant in acute CHS, but not UV-susceptible C3H/HeN mice, indicating that immunologic pathways involved in chronic CHS is different between acute and chronic CHS. Adoptive transfer of T cells from UV-exposed, repeatedly challenged animals, but not of T cells from non-UV-exposed mice, results in successful transfer of the immunosuppressive phenotype to naïve animals, strongly suggesting that UV-mediated suppression of chronic CHS is, at least in part, mediated by induction of suppressor T cells capable of suppression of acute CHS. To further elucidate whether already established chronic CHS is prevented by those suppressor T cells, T cells obtained from UV-tolerised mice in chronic CHS or from non-UV-exposed mice showing immediate ear swelling were transferred to animals that were already repeatedly DNFB-challenged and thus were showing the immediate ear swelling response. While transfer of T cells from non-UV-exposed animals results in no prevention of the immediate ear swelling response, the immunosuppressive phenotype of chronic CHS is partially, but significantly reproduced in animals that have shown immediate ear swelling before the transfer. Together, our present study demonstrates that T cells from UV-tolerized-mice in chronic CHS may act as suppressor T cells both for acute and for chronic CHS, thereby suggesting the presence of a new class of suppressor T cells.

284

UVA and UVB Differentially Modify the Tyrosine Kinase Profile of Normal Human Keratinocytes and Induce the Expression of the Tyrosine Kinase abl-2

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Exposure of human skin to UV induces major changes in the genetic program of the exposed cells leading to immediate and long-term skin changes. Although it can be assumed that UV-induced modifications of signal transduction involving protein tyrosin kinases (PTKs) regulate these processes, details as to the specific changes in PTK expression after UV exposure are unknown. To investigate PTK expression in normal human keratinocytes (HNK) we employed a reversed transcriptase-PCR approach using degenerate primers derived from the conserved catalytic domain of PTKs. PCR products were cloned and PTKs from randomly picked colonies (up to $n = 90$ per screen) were identified by sequence analysis. PTK profiles of sham-irradiated, UVA (filtered metal halide lamp, 60 J per cm^2), and UVB (filtered metal halide lamp, 256 mJ per cm^2) treated HNK were analyzed 7 h after exposure. Real time PCR with specific primers was used to track the kinetics of PTKs identified with the initial screen. We identified 14 PTKs including 3 receptor kinases (axl, cak, fgfr2) and 11 nonreceptor kinases (abl1, abl2, lck, map4k2, fyn, yes, src, cnk, ptk6, mstr1, jak1). Differential screening revealed a further induction of abl2 expression by UVA. At 7 h after exposure UVB had no influence on abl2 but predominantly induced the expression of the receptor kinases of the axl-family. Both treatments lead to a down-regulation of src-family kinases (src, fyn, yes). Overall evaluation revealed that UVA predominantly stimulates expression of nonreceptor kinases and inhibits receptor PTKs, while UVB had the opposite effect. The differences reached statistical significance at $p < 0.0001$ (χ^2 test). Real time PCR revealed that abl2 expression peaked at 16 h after exposure to UVA and reached again baseline at 24 h. UVB induced a late induction at 16 h and 24 h after exposure. We conclude that regulation of PTK expression is part of genetic program that mediates late effects of UVA and UVB. The observed effects are wavelength dependent and affect PTKs which are involved in the regulation of gene transcription, cell death, and proliferation.

285

UVA and UVB Irradiation Decreases the Expression of CD44 and Hyaluronate in Mouse Epidermis

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CD44 is a polymorphic transmembrane glycoprotein and the principal cell surface receptor of hyaluronate (HA). The expression of CD44 in mouse epidermis is confined to the basal, spinous and granular layers. HA, the major component of the extracellular matrix, is a high molecular weight glycosaminoglycan and present mainly in dermis and also in epidermis. HA is capable of immobilizing the water in the tissue and therefore of changing the dermal volume and elasticity, and maintains the extracellular space. The distribution of HA in the skin shows a parallelism to that of CD44. In a recent study we have shown that two major functions of CD44 in the mouse skin are the regulation of keratinocyte proliferation in response to extracellular stimuli and the maintenance of local HA homeostasis. Our previous studies have shown that UVA but not UVB irradiation generated an oxidative stress in SKH1 hairless mouse skin. In this study we examined, by anti-CD44 antibody and hyaluronate binding protein (HABP) stainings, the effect of UVA and UVB irradiation on the expression of CD44 and HA in mouse epidermis. Immunostaining of vehicle-treated back skin of SKH1 hairless mice revealed the standard membrane localization of CD44 and HA in basal and suprabasal keratinocytes. CD44 expression was significantly reduced in the membrane and became cytoplasmic 2 h after UVA (10 J per cm^2) or UVB (1 J per cm^2) irradiation and reconstituted within 8 and 24 h for UVA and UVB, respectively. HA showed a similar pattern of decrease and reconstitution. The importance of the decrease of epidermal CD44 and HA in the mediation of UV-induced deleterious effects and in the generation of oxidative stress induced by UVA irradiation is yet to be elucidated.

286

Mouse Epidermal Vitamin A Reconstitution after UV-Induced Depletion is Oxidative Stress Dependent

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We previously showed that epidermal vitamin A (retinol and retinyl esters) from hairless mice was strongly decreased following an acute UVB exposure (Dermatology 199 : 302–307,1999), and that a pretreatment with topical natural retinoids (retinol, retinal and retinoic acid) prevented a UVB-unduced epidermal hypovitaminosis A (Photochem. Photobiol. 71:in press, 2001). Here, using the same mouse model, we studied the effects of UVA on epidermal vitamin A content and lipid peroxidation (LPO), as well as the the putative prevention of both by topical antioxidants. Vitamin A was assayed by HPLC, and lipid peroxides were assayed by ferrous oxidation followed by orange xylenol complexometry. An acute exposure to UVA completely depleted epidermal vitamin A with ED_{50}s of 0.25 and 0.5 J per cm^2 for retinyl esters and retinol, respectively; these values were 0.1 J per cm^2 for both retinoids with UVB. A single UVA exposure induced a dose-dependent epidermal lipid peroxidation ($ED_{50} = 3.5$ J per cm^2) giving rise to 55.4 ± 4.2 nmol lipid peroxides per g at 20 J per cm^2, while UVB, up to 2 J per cm^2, did not increase the basal concentration of 6.7 ± 0.9 nmol lipid peroxides per g. Topical menadione (vitamin K3) also induced a significant (2.5-fold) increase of LPO, without affecting vitamin A content. A pretreatment with the natural antioxidant α-tocopherol did not inhibit UV-induced vitamin A depletion, but it prevented the increased LPO induced by UV or menadione, and accelerated the reconstitution of vitamin A. Thus (i) acute UVA induced both epidermal vitamin A depletion and LPO (ii) UVB induced only vitamin A depletion (iii) topical menadione induced only LPO, and (iv) topical antioxidants prevented UV-induced LPO, without protecting vitamin A, but accelerated vitamin A reconstitution. This indicates that UV-induced epidermal vitamin A depletion is not mediated by oxidative stress, but the latter affects vitamin A reconstitution following UV irradiation.

287

Effects of Cell Differentiation on the SOD Activity and UVB-Induced Cytotoxicity in Cultured Human Keratinocytes

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We examined the effects of calcium (Ca^{2+}) concentration of culture medium on the SOD activity and UVB-induced apoptosis in cultured human keratinocytes in order to investigate the relationship between Ca^{2+} dependent cellular differentiation and antioxidant capacity. Human keratinocytes were incubated in high Ca^{2+} concentration (> 1 mM) or low Ca^{2+} concentration (< 0.1 mM) DMEM. Then, we measured SOD activity and both Cu,Zn- and Mn-SOD activities in keratinocytes. Furthermore, after incubation in high or low Ca^{2+} DMEM, human keratinocytes were irradiated with UVB. Total SOD and Cu,Zn-SOD activities in keratinocytes cultured in low Ca^{2+} DMEM were significantly lower than in keratinocytes cultured in high Ca^{2+} DMEM, whereas Mn-SOD activity was not affected. LDH leakage from keratinocytes cultured in low Ca^{2+} DMEM and the percentage of apoptotic keratinocytes cultured in low Ca^{2+} DMEM were significantly higher than that of keratinocytes cultured in high Ca^{2+} DMEM following UVB irradiation. These results indicate that well differentiated epidermal cells have higher Cu,Zn-SOD activities and resistance to UVB-injury, suggesting that keratinocytes in outer layer have stronger protective ability to exogenous stresses.

288

Phototoxicity of Curcuma Longa Extract in Cultures of Human Keratinocytes

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The use of psoralens and UVA (PUVA) is an effective treatment of severe psoriasis and other skin diseases. However, PUVA therapy holds some risk of development of skin cancer. Searching for alternatives we investigated the effect of yellow *Curcuma longa* extract (ZCL8) combined with both, UVA or visible light on the human keratinocyte cell line HaCaT and on normal skin derived human keratinocytes *in vitro*. Uptake studies were carried out measuring the absorbance of methanolic extracts of the cells after treatment with 10 μg per mL ZCL8. Growth inhibition was measured by BrdU incorporation. The intracellular distribution was investigated by confocal laser scanning microscopy making use of the low fluorescence activity of *Curcuma longa* compounds. The absorbance maximum of ZCL8 was at approximately 430 nm. The uptake studies revealed a maximum uptake of ZCL8 by human keratinocytes within about one hour. Afterwards a slow bleaching of the intracellular colored ZCL8 compounds was observed indicating a metabolic destruction and/or conversion. The fluorescent ZCL8 compounds showed a granular, perinuclear cytoplasmatic location 40 min after addition to the cell culture medium. There was no detectable fluorescence in the cell nuclei even after UVA irradiation and after an incubation time of 4 h. Using the BrdU incorporation technique no detectable phototoxic effects occurred after treatment with ZCL8 combined with UVB light. However, ZCL8 showed clear phototoxic properties in both the human keratinocyte cell line (HaCaT) and in the cultures of normal human epidermal cells in combination with UVA, UVA1 or visible light. The degree of phototoxicity was comparable with that of psoralens. All 3 effective light sources caused growth inhibition in the presence of concentrations as low as 1–2 μg per mL of ZCL8. Investigations of the essential preincubation time for ZCL8 showed that 1 h of preincubation was sufficient to obtain optimal phototoxic effects in the cultures of human keratinocytes. These findings could contribute to a new photo therapeutic treatment strategy with low risk. Additional investigations with regard to the effects on the immune system, to the DNA integrity, and to the underlying mechanisms of growth inhibition are currently carried out.

289

Nitric Oxide and PGE_2 Mediate UVB-Induced Erythema

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Prostaglandin E_2 (PGE_2) and nitric oxide (NO) have been implicated in the generation of UVB-induced vasodilatation, but their relative contributions are unclear. We explored the effects of inhibiting synthesis of PGE_2 and NO with indomethacin and L-NAME on vasodilatation induced by 1, 2 and 4xMED of UVB. Single applications of 1% indomethacin gel immediately after irradiation significantly suppressed strong erythema from 2 and 4xMED but had little or no effect on weak erythema (1MED). Repeated applications every 12 h did not increase the inhibition. The slope of the UVB erythema dose–response curve was significantly flattened by indomethacin. Twenty-four hours after irradiation with 4xMED, mean PGE_2 levels in suction blisters were 27.2 ng per mL (SE 11) compared with 8.6 ng per mL in unirradiated skin ($n = 25$; $p < 0.01$). PGE_2 levels in dermal tissues, sampled by microdialysis (depth 0.6 ± 0.1 mm), were 310 pg per mL (SE 123) and 237 pg per mL (SE 88) in irradiated and unirradiated skin, respectively ($n = 7$, NS). Nitric oxide also made a significant contribution to UVB-induced erythema. UV-erythema was inhibited by L-NAME in a dose-related fashion with 2 mM L-NAME causing total abolition of the response. In contrast to indomethacin, L-NAME shifted the UVB erythema dose–response curve in parallel. L-NAME was effective at all time points up to 48 h suggesting that nitric oxide (NO) was produced continuously. NO was undetectable in suction blister fluid but in dermal microdialysate NO was present at 44.3 ng per mL (SE 6.2) following UVB compared with 26.0 ng per mL (SE 8.0) in unirradiated skin ($p < 0.05$), approximately 1000 times the molar concentration of PGE_2. These findings confirm PGE_2 and NO to be mediators of UV-induced erythema. They also show that there is prolonged synthesis of both mediators within the erythemal response and that synthesis of NO is induced by lower doses of UVB compared with that of PGE_2. It remains to be shown whether PGE_2 is generated as a consequence of activation of Cyclo-oxygenase by NO.

290

UV-Induced TNFα Production is Influenced by the Application of Sunscreens

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Human *in vitro* methods to test the immunoprotective effect of sunscreen are rare. We used human skin explants to investigate the effect of two different sunscreens (containing UVB-filter alone or UVB and UVA-filters) on the production of TNFα and interleukin 10 after irradiation with different doses of solar simulated UV. Skin explants of the thigh were obtained from 12 different individuals undergoing plastic surgery. First the skin was divided into 2 pieces of equal size, each half was treated with placebo cream or a sunscreen. Each of the two pieces was again divided into 4 parts which were irradiated with a solar simulator with 0.5, 1, and 2 minimal erythema dose equivalent (MEDE) multiplied by the sun protection factor (SPF) of the placebo or of the sunscreens. One quarter of each group was sham irradiated. After irradiation at least three 6-mm skin biopsies were cut of each part and floated on RMPI medium for 48 h. The concentration of the soluble mediators in the medium was measured by ELISA. TNFα production increased with increasing irradiation dose. Both sunscreens significantly prevented the production of TNFα compared to the placebo treated group. SSC-UVAB showed a significant increase of TNFα already in 1 MEDE group compared to the sham irradiated group, whereas in the placebo and SSC-UVB treated groups TNFα significantly differed only between sham irradiated and 2MEDE groups. Both sunscreens reduced the production of TNFα after irradiation with 2 MEDE about 50% compared to the placebo treated group. The production of interleukin 10 did neither correspond to the irradiation nor to the treatment groups. Skin explants can be used to measure the UV induced TNFα and interleucin 10 production. Using this *in vitro* method sunscreens containing organic UVB-filter or UVB and UVA-filters showed a protective effect on the production of TNFα comparable to their SPF.

291

UV-Induced Cytochrome *c* Release During Apoptosis is Mediated Primarily via Death Receptor Activation and Free Radical Formation but Mostly Independent of UV-Induced DNA Damage

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During UV induced apoptosis nuclear DNA damage as well as direct activation of death receptors play a major role, both pathways beeing independent of each other. Additionally release of cytochrome *c* (cyto*c*) from mitochondria into the cytoplasm is observed after UV radiation, another important event during apoptosis. Cytoplasmic cyto*c* together with ATP and Apaf-1 activates the effector cysteine protease caspase-9, resulting in execution of apoptosis. We tried to elucidate whether cyto*c* release during UV-induced apoptosis is a consequence of DNA damage, of death receptor activation or of a third pathway. Western blot analysis revealed cyto*c* release into the cytoplasm of UV-irradiated HeLa cells starting 8 h after UV exposure. Inhibition of death receptor clustering by UV by irradiating cells at 4°C blocked cyto*c* release partially, whereas removal of DNA damage by enhancing DNA repair via photoreactivation did not affect cyto*c* release at all. This indicates that UV-induced cyto*c* release seems to be DNA damage independent, but mediated via cell death receptor activation. During death receptor signaling caspase-8 induces cyto*c* release via cleavage of Bid. Consequently, UV-induced Bid cleavage was inhibited in cells exposed either at 4°C or in the presence of zIETD the specific inhibitor of caspase-8. The only partial inhibition suggested other pathways to be involved as well. Addition of the free radical scavenger pyrrolidinedithiocarbamate significantly suppressed UV-mediated cyto*c* release and consequently apoptosis. Hence, these data indicate that cyto*c* release during UV-induced apoptosis is primarily mediated by cell death receptor activation and free radical formation, but mostly independent of DNA damage.

292

DNA Damage Mediates Downregulation of the UV-Repressible Serpin Hurpin (PI13) in HaCaT Keratinocytes

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Epidermal keratinocytes are the primary target of midrange ultraviolet part of terrestrial sunlight (UVB, 290–320 nm). Analysis of the resulting UV response at the transcriptional level by differential display RT-PCR identified a large group of genes demonstrating a down regulation following UVB irradiation. Among those UV-repressible genes, a novel, keratinocyte associated serine protease inhibitor (serpin) termed hurpin (HaCaT UV-repressible serpin) has been identified.In this study we try to unravel the molecular mechanism underlying the observed down-regulation of hurpin. Based on the knowledge of UVB-mediated effects the irradiation induced DNA-damage itself could be one of the mediators of the repression. To analyze the role of UV-mediated DNA-damage in the repression of hurpin we used two different set-ups. In the first we enhanced the repair capacity of the irradiated cells by supplying them with the DNA-repair enzyme Photolyase. In the second we preserved the DNA-damage by blocking the DNA-repair by treatment with Aphidocolin. Following these treatments we analysed the expression of Hurpin in both treated and untreated HaCaT cells by RT-PCR analysis. Enhancing DNA-repair through Photolyase led to a less pronounced hurpin repression compared to the expression pattern following UVB irradiation alone. On the other hand the preservation of the UV-induced DNA damage by incubation with the repair inhibitor Aphidocolin resulted in a more prominent and longer persistent down-regulation of hurpin. These findings suggest that UVB induced DNA damage plays an important role in the regulation of the expression of hurpin. According to previous observations hurpin might be involved in proliferation and differentiation of keratinocytes through a possible antiapoptotic function. Such a function would be in good accordance with the observed overexpression of hurpin in psoriatic skin lesions compared to healthy skin. Notably psoriasis is responsive to UV-irradiation. Further investigation of the mechanisms underlying the regulation of hurpin after UVB irradiation could lead to new therapeutical approaches in diseases characterized by hyperproliferation of keratinocytes, such as psoriasis.

293

Epidermal Response to UV-Induced DNA Damage

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In order to determine levels of UV-induced DNA damage and p53 expression in epidermis at different sun-exposed sites, chronically sun-exposed and nonexposed skin were analyzed in the same set of individuals. Healthy volunteers were irradiated with artificial UVA + UVB, with and without photo-protection. Skin from same individuals were also analyzed after six summer weeks in Sweden. The total UV-dose received from natural sun was measured. Punch biopsies were taken 24 h after UV-irradiation and after 6 summer weeks, and immunohistochemically stained for thymine-dimers and p53 protein. Non-exposed skin showed more DNA damage compared to chronically sun-exposed skin after UV-irradiation. Photo-protection reduced levels of DNA damage and p53 protein. A correlation between amount of p53 immunoreactive keratinocytes and UV-dose received during 6 summer weeks was found. Frequently sun-exposed skin is located in cosmetic areas, why *in vivo* experiments are difficult to perform. We developed a skin organ culture model, where UV-responses seen *in vivo* can be reproduced *in vitro*. Human skin explants from chronically sun-exposed and nonexposed skin were irradiated with artificial UVA + UVB, with and without sunscreen (SPF 15). Kinetics of UV-induced DNA damage, p53 response and repair was analyzed by immunohistochemistry. A large amount of TT-dimers was observed 4 h after UV-irradiation followed by an accumulation of p53. TT-dimers were gradually repaired during incubation time. A shown *in vivo*, sunscreen had the ability to reduce the formation of TT-dimers and induction of p53 protein normally found in nonprotected skin after UV-irradiation. In conclusion, data demonstrates inter- and intraindividual variability in responses to UV-induced DNA damage including a comparison between chronically sun-exposed skin and nonexposed skin. DNA repair appeared more efficient in chronically sun-exposed skin compared to nonexposed skin. *In vitro* results suggest that organ cultured skin provides a valuable tool for studies of UV-induced epidermal responses in chronically sun-exposed skin.

294

Analysis of Specific DNA Damages Induced by UVA and UVB Radiations on Human Skin

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In addition to biological effects on human skin such as inflammation, immunosuppression and premature photoaging, ultraviolet (UV) radiations can initiate skin photocarcinogenesis through induction of DNA damage. Both UVB and UVA radiations have been described as mutagenic. However, the processes by which they alter DNA are different. UVB light is directly absorbed by the DNA bases and induces predominantly dipyrimidine photoproducts such as cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4) pyrimidone adducts ((6–4)PP). At higher wavelengths corresponding to UVA, DNA absorption is very weak. However, UVA and to a lesser extent, UVB may interact with other cellular chromophores giving rise to reactive oxygen species through photosensitization processes. DNA and particularly guanine could be the target of ROS. As a result 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) has been shown to be mainly generated. In order to determine the specific contribution of the different ranges of UV radiation to the induction of DNA damages, human skin samples were exposed to broad sources of UVB (312 nm), UVA (365 nm) and both UVB + UVA. Subsequently DNA was extracted and purified prior its analysis. Thus, the content of DNA in CPD and (6–4) PP was estimated by immuno-dot-blot assays using specific antibodies. In addition, the formation of the latter adducts and of the 8-oxodGuo, was singled out using the highly resolutive HPLC-MS/MS method. It was also established the noncytotoxic dose ranges of UVA (5–12.5 J per cm^2) and UVB (0.01–0.1 J per cm^2) that allow the formation of a maximum of photodamage in cutaneous DNA. CPD appear to be the major lesions induced by both UV radiations, UVB light being however, more efficient than UVA to generate these photoproducts. UVA and UVB radiations are both able to induce the 8-oxodGuo which is, however, more effective at higher wavelengths. The validation step being achieved, we have used both the skin system and the above analytical methods to evaluate the photoprotection efficiency of sunscreens and the antioxidant activity of products.

295

The Mutagenic Effects of Long-Wave Ultraviolet A in Human Skin; Studied by Analysing the p53 Gene in Single Cells

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Skin cancer is the most common cancer in the western world, and the incidence is increasing. Ultraviolet B radiation is considered the major cause of skin cancer. However, several findings indicate that also ultraviolet A radiation may play a role in carcinogenesis of the skin. It has previously been shown that repetitive suberythemal doses of long-wave ultraviolet A on healthy human skin induced an increase of p53 positive cells in epidermis. We now examined if long-wave ultraviolet A had mutational effects on *p53* in human skin. Three healthy individuals were irradiated 6 times with physiological fluences (40 J per cm^2) of long-wave ultraviolet A on unexposed skin. Biopsies taken after the last exposure and five days after the last exposure were analysed. Microdissection of p53 immunopositive single cells were followed by a modified multiplex amplification and sequence analysis of exon 4–11 of the *p53* gene. Three mutations were found, one in codon 231 of exon 7 and the other two in introns. All were C?A mutations, typical for UVA. This gives an average of 1 mutation per 8700 bases or 1 per 12 cells. It correlates well with the earlier findings of ultraviolet A signature mutations in normal human sunexposed skin. Our findings indicate that this amount of mutations represents the mutation load of long-wave ultraviolet A resulting from normal sun exposure.

296

Adalimumab, a Fully Human Anti-TNF-α Monoclonal antibody (MoAb), Reduces the UVB Induced Expression of c-jun and Phosphorylated c-jun *In Vivo*

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TNF-α is known to play an important role in UV induced immunomodulation and photodamage. It plays a role in UVB mediated induction of apoptosis and is a strong inducer of the JNK pathway, which eventually leads to the loss of dermal collagen and elastin content. Recently chimeric anti-TNF-α has been introduced as a therapy for rheumatoid arthritis. The present study focuses on the effect of anti-TNF-α on apoptosis and c-Jun/phosphorylated c-Jun induction, aspects which have been claimed to be TNF-α dependent and which could affect photoaging and carcinogenesis. Twelve patients with rheumatoid arthritis were included and irradiated with 2 MED broad band UVB before and after administration of 0.5 mg per kg anti-TNF-α monoclonal antibody. Biopsies were taken 24 h after each irradiation and frozen sections were stained for p53, c-Jun, phosphorylated c-Jun and sunburn cells. No significant changes were observed in the expression of p53 and sunburn cells after treatment with anti-TNF-α, whereas a significant decrease in c-Jun and phosphorylated c-Jun expression was noted ($p = 0.0250$ and $p = 0.0431$, respectively). The reduction of c-Jun and phosphorylated c-Jun may lead to a decrease in expression of matrix metalloproteinases and suggests a protective effect on photoaging. Anti-TNF-α at therapeutic doses decreases c-Jun/phosphorylated c-Jun expression whilst leaving apoptosis and p53 expression unaffected.

297

Effects of UVB Radiation on Migration and Activation of Langerhans Cells in PLE Patients and Healthy Volunteers

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UV radiation suppresses cellular immune reactions in healthy individuals. This suppression is associated with the disappearance of Langerhans cells (LC) from the epidermis, and is thought to prevent illicit immune reactions in UV -irradiated skin. Failure in this suppressive response may lead to adverse skin reactions, such as polymorphic light eruption (PLE). In contrast to what is observed in healthy volunteers, we found that the LC of PLE patients did not disappear after overexposure (6 MED) of the buttock skin to UVB. Subsequently, we established that the disappearance of LC in healthy individuals is due to migration. This led us to the hypothesis that LC in PLE patients are defective in their migratory response. Furthermore, we speculated that the 'UVB-resistant' LC might become activated and present antigens at the wrong location (viz. the epidermis) thereby contributing to the pathogenesis of PLE. To test these hypotheses, we exposed buttock skin of PLE patients and healthy volunteers to 6 MED UVB, and suction blisters were raised 16–18 h later on the exposed and on an unexposed area of the skin. Blister fluid and blister roofs were collected and stained immunohistochemically for CD1a (LC). Skin biopsies were taken 24 and 48 h after the exposure and stained for activation markers (CD86, CD40, ICAM-1, HLA-DR) and for the dendritic cell maturation marker CD83. Migrating LC were found in the blister fluid of healthy volunteers as well as PLE patients. The decline in the number of LC in blister roofs of PLE patients was, however, only very moderate in comparison to the almost complete depletion of LC in blister roofs of healthy volunteers. The ''UVB-resistant'' LC in PLE patients, and the few epidermal LC in UVB-exposed skin of healthy volunteers, did not express CD86, CD40, ICAM-1 or CD83, but did strongly express HLA-DR, in contrast to LC in the unexposed skin. Our data show that LC in PLE patients are not defective in their migratory response to UV exposure, and that the remaining epidermal LC do not become fully activated or matured. The enhanced state of HLA-DR may, however, cause a strong, illicit antigen presentation by LC in UV-irradiated skin of PLE patients.

298

Photodynamic Therapy Induces Neovascularisation in Psoriatic Plaques

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The effect of photodynamic therapy (PDT) was evaluated in eight psoriatic patients. One plaque was selected in each patient and treated once weekly with PDT 10–30 J per cm^2 2–5 times. The result was evaluated clinically by using a modified Psoriasis Area and Severity (PASI) score (maximal score/patient = 9) and a Visual Analogue Scale (VAS), ranging from 0 to10, to assess pain during treatment. Skin biopsies were taken before treatment, after two treatments and after completion of treatment, and evaluated by immunohistochemistry. Median PASI scores were reduced from 7 (range 5–9) to 2 (range 0–3) following treatment. Pain during PDT was assessed as 7 (range 3–10). The number of dermal vessels, identified by antibodies against Factor VIII, markedly increased in five of eight patients. Before treatment, there was a moderate infiltrate of $CD4^+$ cells and a few $CD8^+$ cells in the dermis. The EGF receptor was displayed throughout epidermis, keratin 16 suprabasally, involucrin from stratum granulosum to the lower spinous layers and filaggrin in stratum granulosum with focal absence. Following treatment the EGF receptor was displayed throughout the epidermis in most of the specimens, whereas the expression of cytokeratin 16 was markedly decreased. The expression of involucrin was not seen as deep in the spinous layers as before PDT. Filaggrin was expressed throughout stratum granulsom and a weak staining was sometimes present in a few granular layers. The number of $CD4^+$ and $CD8^+$ dermal cells decreased. PDT improved psoriasis and induced dermal neovascularisation. The mechanism of the neovascularisation is unknown, but may be a recovery phenomena.

299

Antinuclear Antibodies in Patients with Polymorphous Light Eruption (PLE): An 8.5-Year Follow-Up Study

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Previous studies have shown that elevated titers of antinuclear autoantibodies (ANA) may occur in 4–19% of patients with polymorphous light eruption (PLE). Whereas in some of these patients a diagnosis of lupus erythematodes (LE) was finally established upon further examination, in the prevailing majority the diagnostic criteria of LE were not met. The present long-term follow-up study was performed to investigate whether the presence of ANA in PLE patients is associated with an increased risk of eventual progression into LE. The study cohort comprised 472 PLE patients who were seen at our phototherapy unit between 1986 and 1999 and had been tested for the presence of ANA. ANA positivity was defined as an ANA titer of = 1 : 80. All ANA positive patients were asked to attend for follow-up examination that included a complete blood cell count, serum and urine chemistry, serum protein electrophoresis, total Ig, C3c, C4 and CH50 levels, ANA, and antibodies to SS-A/Ro, SS-B/La, U1 RNP, Sm, Scl-70, Jo-1, dsDNA, histones and centromeres. The median age of all 472 patients was 34 years (range, 12–75) and the median disease duration 8 years (range, 1–60). Out of all patients 55 (11.7%) were found to be ANA positive on one or several occasions, two of them also had antibodies to SS-A/Ro. 39 (70.9%) of all ANA positive patients were available for a thourough follow-up examination. Their median age and disease duration was 41 years (range, 23–73) and 15 years (range, 5–34), the median follow-up period was 8.5 years (range, 1–14). 25 patients showed persistence of ANA positivity with a median titer of 160 (range, 80–640), whereas in 14 patients ANA titers had reverted to normal levels. Clinical and laboratory examination of all 39 patients including the 2 patients with antibodies to SS-A/Ro did not reveal any finding suggestive of LE. In addition, in the latter 2 patients the eruption was short-lived, histopathology of lesional skin was indicative for PLE, and direct IF as well as lupus band test were both negative. In conclusion, we found an increased prevalence (11.7%) of moderately elevated ANA in a cohort 472 PLE patients. Antibodies to SS-A/Ro were negative in all but two patients who lacked further evidence of LE. None of the ANA positive patients developed LE within the follow-up period. These findings indicate that PLE has no tendency to progress to LE.

300

The Time Course of UVB-Induced Erythema in Red-Heads

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The melanocortin 1 receptor gene (chromosome 16q24.3) is currently the only gene identified that explains substantial phenotypic variance in human pigmentation. In people with MC1R variants associated with red hair, fair skin and increased sensitivity to burning from UV radiation is described. To further delineate the red-hair phenotype, we have objectively followed the duration and intensity of cutaneous erythema induced by UV-B radiation (Oriel Xenon-arc source) for 21 days in 10 red-headed subjects (homozygous loss of function mutations) and 10 nonred controls using contact laser Doppler flux (LDV) methodology (Moor instruments). Triplicate LDV readings were made on 7 separate occasions over a 21-day study period. The results were analysed using Mann–Whitney-*U*-test and Students' *t*-test on Arcus Quickstat. Following duplicate serial dosing of UVB on the lower back (9 doses of 5–112 mJ per cm^2 in √2 increments), maximal erythema was seen at 1–2 days depending on the dose of UVB delivered. There was no significant difference in the intensity of maximal erythema induced in either group ($p = 0.771$). At the end of the study period, both groups contained individuals with persistent erythema, but there was no significant difference between red-heads and nonred controls ($p = 0.331$). However, when area under the curves were obtained from serial data and the mean areas calculated, red headed individuals demonstrated a 2–4 fold increased mean flux in response to 40 and 28 mJ per cm^2 of UVB ($p = 0.0026$ and $p = 0.0175$, respectively). Sustained erythema to a single exposure of UVB of 5–112 mJ per cm^2 is not a feature of red-headed-individuals. However, red-heads develop more total erythema when exposed to 28 and 40 mJ per cm^2 of UVB than nonred controls when followed over time (21 days).

301

Reproducibility of Irritant Patch Tests Over Time

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Variation in response to inflammatory stimuli may be under primarily genetic or environmental control. In man as compared with model organisms the position is not clear. One way to get a handle on partitioning the variance between genetic and environmental causes is to look at variation over time within the same individuals and between individuals. We assessed the response of increasing concentrations of broadband ultraviolet B radiation ($2^{1/3}$ increments from 119 to 300 mJ cm^{-2} – Phillips PLS 9 W/12), doubling doses of anthralin (0.005–0.08%) and sodium dodecyl sulphate (0.5–8%) in 15 healthy volunteers over time. Patch tests were applied in duplicate to the upper back for 24 h using 8 mm Finn chambers (Epitest Ltd, Finland). The same irritants and inflammatory stimuli were then applied 2–3 months later, again in duplicate to the adjacent skin. The inflammatory response was measured objectively using a reflectance spectrophotometer, a Lisca scanning laser Doppler and the Moor contact laser Doppler. Statistical analyses of data, using Excel, produced dose–response curves and the gradient of the curves were then compared. There was a striking lack of reproducibility over time particularly with the SDS (greater than 50% variation) compared with ultraviolet B (less than 15% variation). The amount of variation within individuals on the same day was much less for all irritants compared with variation over time. The variation amongst test substances could be ranked as UVB (8%) less than anthralin (12%) less than SDS (33%). Although our results are preliminary, they suggest that there are large temporal influences on the inflammatory system. Such results would suggest limits to genetic determinants of cutaneous inflammation, at least in an experimental system.

302

Demonstration of a Clinically Relevant Sensitization to House Dust Mite Allergen in Prick and RAST Negative Intrinsic Atopic Dermatitis Patients with the Atopy Patch Test

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Atopic dermatitis (AD) is a clinically defined, highly pruritic skin disease frequently associated with IgE responses against aero- and food-allergenes. A so-called intrinsic type of AD (IAD) has been delineated from the more common extrinsic AD (EAD) by normal serum IgE levels, negative RAST tests and negative immediate-type skin reactions towards environmental allergenes. Since in IAD, a clinically relevant sensitization against these allergens cannot be determined by standard prick tests or RAST, there is a need for other test procedures. Here we report 2 key patients in which the recently standardized atopy patch test (APT) was employed to confirm the clinically suspected role of house dust mite allergen for their AD. A 24-year-old optician and a 59-year-old masseur with clinically characteristic AD lesions both had a negative family history for atopy. The two female patients both experienced an itch sensation after house dust exposure, which was followed by a flare up of their AD lesions. The allergological workup did not show any positive prick test or RAST results, and the total serum IgE was 23 kU per liter and 55 kU per liter, respectively. A diagnosis of IAD was made and an APT was performed in the two patients, which showed strongly positive results for the house dust mite allergen D. pter. in a test concentration of 200 IR. Epidermal Dendritic Cell Phenotyping (EDCP) is a recently standardized diagnostic procedure based on the quantitative evaluation of Langerhans cells and inflammatory dendritic epidermal cells (IDEC) of inflamed skin. The naturally developed lesions of EAD are characterized by an increased number of IDEC with a high expression of FceRI on their cell surface. The EDCP of our two patients' APT reactions showed an unusually high number of IDEC, but a low FceRI-expression – a pattern previously seen in naturally developed IAD lesions. Our conclusion is that the APT may become an important diagnostic tool especially in patients with IAD where the standard prick tests and blood analyses do not identify those allergens relevant to the clinical course of disease.

303

Atopic Dermatitis and Concomitant Disease Patterns in Children Up to 2 Years of Age

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There are few prospective studies of atopic dermatitis and coexisting diseases such as respiratory infections, in children up to two years of age. Using annual questionnaires, we studied the two-year-prevalence of atopic dermatitis and concomitant symptoms of other atopic diseases and respiratory infections in 0–2-year-old children in a prospective birth cohort of 4089 children. There was an increased risk of asthma (RR 1.45, 95% CI 1.16–1.80), allergic rhinoconjunctivitis (RR 2.25, 1.77–2.85), adverse reactions to foods (RR 3.20, 2.83–3.62), and urticaria (RR 2.04, 1.80–2.31) as well as otitis (RR 1.13, 1.05–1.21), more than one pneumonia during the first and/or second year of life (RR 2.17, 1.14–4.15) and the use of antibiotics at least twice yearly (RR 1.29, 1.07–1.56) in children with AD as compared with those without. The risk of atopic disease manifestations, but not respiratory infections, was higher with onset of atopic dermatitis during the first year of life than during the second. Already during their first two years of life, children with AD have a significantly increased risk not only of other atopic disease manifestations, but also of respiratory infections manifested in an increased rate of otitis, pneumonia and use of antibiotics.

304

The Prevalence of Atopic Diseases and the Level of IgE in Patients with Systemic Lupus Erythematosus

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The coexistence of atopic diseases (AD) and systemic lupus erythematosus (SLE) as well as the observations regarding the elevated level of IgE in SLE patients remain controversial. Therefore the aim of the study was to investigate the prevalence of atopic diseases among patients with SLE and the serum level of total IgE. The group of patients consisted of 39 patients (36 female, 3 male) aged 30–50 years (mean = 38.5). All the patients were in inactive phase of SLE. The activity was evaluated according to Liang score. The diagnosis of atopic diseases was based on interview, physical examination and additional tests. Total IgE concentration was determined in the sera by ELISA using commercially available kits. The control group consisted of 31 healthy persons, age and sex-matched. Atopic diseases were present in 26% of patients. The most frequent diagnosis was: allergic rhinitis (9/39), atopic dermatitis (2/39), and allergic conjunctivitis (1/39). More than 1 atopic disease was diagnosed in 2 patients. The mean IgE concentration in SLE patients was 204 IU per mL (Me = 49 IU per mL) and 37 IU per mL (Me = 24 IU per mL) in the controls. The difference was statistically significant ($p = 0.02$). In SLE patients with concomitant atopic diseases IgE concentration was 496 IU per mL (Me = 169 IU per mL) and in the patients without AD 104 IU per mL (Me = 38 IU per mL). There were no statistically significant differences ($p > 0.05$) between the IgE concentration in SLE patients without AD and the controls. The higher level of IgE in SLE patients was connected with the coexistence of atopic diseases. In conclusion, it seems that the prevalence of atopic diseases in SLE patients and the IgE level in inactive phase of the disease is not different from the general population.

305

Treatment of Chronic Plaque Psoriasis by Targeting CD45RO+ Memory-Effector T Lymphocytes: A Randomized Phase II Trial of Alefacept (Amevive™)

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Chronic plaque psoriasis is characterized by infiltration of CD45RO+ memory-effector T-lymphocytes in the skin. Alefacept (human LFA-3/IgG1 fusion protein, currently being developed under the trade name, AMEVIVE™) selectively binds to CD2 receptors expressed on these T cells. It blocks the LFA-3/CD2 interaction, reduces T-cell activation, and modifies the inflammatory process. A multicenter, randomized, placebo-controlled, double-blind study was conducted to evaluate the efficacy, safety, and quality-of-life (QoL) impact of alefacept in patients with psoriasis. 229 subjects were randomized to receive placebo or alefacept (0.025, 0.075, or 0.150 mg per kg) administered as a 30-second IV injection once weekly for 12 weeks. Efficacy endpoints were assessed at 2- and 12-weeks after dosing and included changes from baseline in PASI and PGA. Alefacept was effective in the majority of patients, with 76% achieving > 50% improvement in PASI scores at anytime after receiving the first dose. A significantly greater percent of patients who received alefacept 0.075 mg per kg IV demonstrated ≥75% reduction in PASI from baseline at 2- and 12-weeks after dosing compared with placebo (33% vs. 10%; $p = 0.02$ and 31% vs. 11%; $p = 0.02$, respectively). Based on PGA, a significantly greater percent of patients in the alefacept group were clear or almost clear of psoriasis at 2 and 12 weeks after completion of therapy ($p < 0.05$ vs. placebo). Alefacept therapy was well tolerated, with no reports of disease rebound after treatment was completed. There was no evidence of an increased risk of infection with alefacept therapy. Of the 229 patients enrolled, 205 completed baseline and follow-up QoL questionnaires. Significant treatment effects were observed; patients receiving alefacept reported greater improvements in the dermatology-specific instruments, DLQI and DQOLS, compared with those who received placebo ($p < 0.05$). In this phase II trial, alefacept produced clinically meaningful and statistically signifcant responses with a favorable safety profile in patients with chronic plaque psoriasis. There are no reports of rapid flares or rebound following discontinuation of therapy. Additionally, when compared with placebo, alefacept was associated with greater improvements in patients' QoL.

306

PUVA Maintenance Treatment for Psoriasis

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Photochemotherapy (PUVA) is a highly effective and widely performed treatment option for patients with severe psoriasis. Like other currently available antipsoriatic agents PUVA is only remittive and most patients relapse within one year after cessation of treatment. One approach aimed at prolonging the disease free interval is maintenance treatment which denotes continuation of PUVA treatment beyond the clearing phase at a lower frequency. Studies performed so far on the efficacy of maintenance treament for preventing early relapse of psoriasis are scarce and have provided inconsistent results. We therefore sought to reinvestigate this important issue by means of a prospective, intrapatient, left-right comparison study. 34 patients with chronic relapsing plaque type psoriasis were included in the study. PUVA treatment for clearing was given 4 times weekly according to the European standard protocol. Thereafter all patients were placed on a halfside maintenance schedule that consisted of two weekly irradiations over 4 weeks followed by one weekly irradiation for another 4 weeks. In all patients complete or near complete clearing was achieved after a mean of 19.7 ± 7.0 exposures, 41.2 ± 16.9 days and a total UVA dose of 55.3 ± 43.2 J per cm^2. The respective values for maintenance treatment were 10.4 ± 2.1 exposures, 47.8 ± 14.1 days and 50.4 ± 48.3 J per cm^2. The mean number of maintenance exposures was less than 12 because 9 patients already relapsed on both half-sides while still under maintenance treatment. Overall, maintenance treatment was disappointingly ineffective in stabilizing the therapeutic outcome of the clearing phase. In only 3 patients (8.8%) a delay in the onset of recurrence of 1.5–2 months was observed. No half-side difference with respect to the time until appearance of new lesions was detectable in the remaining 31 patients (91.2%). The mean time interval until relapse without and with maintenance irradiation was 4.5 ± 3.4 months and 4.6 ± 3.4 months, respectively. Our data demonstrate that short-term maintenance treatment while considerably adding up to the patients' cumulative UVA exposure load is ineffective in preventing early relapse in the great majority of patients and should be avoided.

307

A Three-Month Multiple-Dose Toxicity Study of Alefacept (Amevive™) in Baboons

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The toxicity of alefacept (human LFA-3/IgG1 fusion protein, currently being developed under the trade name AMEVIVE™) was evaluated following repeat IV administration to baboons. Twenty-four baboons were divided into 4 dose groups, with 3 males and 3 females in each group, and randomized to receive sterile saline or alefacept 0.1, 2, or 40 mg per kg administered once weekly for 13 weeks. The 40-mg per kg per week dose is approximately 500 times higher than the clinical dose used in human subjects. On Day 93, one preselected male and female baboon from each group were sacrificed. The remaining animals were evaluated for an additional 7 months and sacrificed on Day 316. Overall, alefacept was well tolerated and no clinical signs of toxicity, infectious compromise, or neoplastic changes were observed during treatment or throughout the 224-day observation period. Alefacept-related changes were limited to dose-dependent reductions in peripheral and tissue-associated T-cell subsets in all 4 dose groups. However, these effects appeared to be saturable, with CD2+ and CD4+ T-cell reductions plateauing at approximately 80% below baseline. Despite the use of doses up to 500 times higher than the clinical dose, T-cell subsets could not be depressed below this threshold. Additionally, T-cell reductions were not significantly different in animals treated with the 40-mg per kg per week dose compared with the 2-mg per kg per week group. By Day 316, peripheral lymphocyte counts had returned to predose baseline levels in the 0.1-mg per kg per week group and continued to recover toward baseline levels in the 2- and 40-mg per kg per week groups. There were no gross abnormalities observed in any animals. Alefacept therapy led to subtle yet distinct reductions in cellularity of T-cell-specific regions of the spleen and the lymph nodes. Tissue changes remained evident in the spleens of the recovery sacrificed animals that had received alefacept 40-mg per kg per week. However, the extent of these changes was less evident than in those animals evaluated on Day 93. Weekly alefacept administration was well tolerated with no signs of toxicity, infectious compromise, or neoplastic changes observed during treatment. The effects of alefacept on peripheral T cells were saturable at 80% below baseline. Additionally, animals receiving the 0.1-mg per kg per week dose, which is representative of the clinical dose, demonstrated complete recovery from reductions in peripheral and tissue-related lymphocyte counts following treatment cessation.

308

Alefacept (Amevive™) Therapy Targets Key Mediators of Psoriasis and Produces Long-Lasting Clinical Response

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The presence of memory-effector T cells (CD4+ CD45RO+ and CD8+ CD45RO+) in the skin stimulates hyperproliferation of keratinocytes, leading to the development of psoriatic plaques. Memory-effector T cells are generated when antigen-presenting cells process antigen, migrate to regional lymph nodes, and interact with naïve T-cells. CD2 is a cell-surface protein that is expressed on all T-cell subsets, but is up-regulated on the surface of memory-effector T cells. LFA-3, expressed on antigen-presenting cells, is the natural ligand of CD2. Interaction of CD2 with LFA-3 costimulates T-cell activation and proliferation and increases cytotoxic T-cell effector function. Alefacept, a fully human fusion protein of LFA-3/IgG1 (currently being developed under the trade name AMEVIVE™) inhibits memory-effector T-cell activation by blocking the costimulatory interaction between CD2 and LFA-3. It also interacts with FcγRIII receptors on natural killer cells and facilitates T-cell apoptosis. Alefacept has a selective immunomodulatory effect that does not interfere with the activity of naïve T-cells or other immune cells, such as B cells. The effect of this targeted action of alefacept on duration of clinical response was evaluated. Patients with chronic plaque psoriasis ($n = 229$) were randomized to receive placebo or alefacept (0.025, 0.075, and 0.150 mg per kg) administered as a 30-second IV injection once weekly for 12 weeks. Efficacy endpoints included Physician Global Assessment (PGA) and changes in PASI from baseline. Upon completion of the study, patients had the option to receive subsequent doses of alefacept in an open-label study, where the time to retreatment was recorded. During the phase II trial, 19 patients were clear or almost clear of disease by PGA 2 weeks after dosing was complete, with 84% maintaining this response during the 12-week follow-up period. An additional 12 patients continued to improve and became clear or almost clear of disease over the 12-week postdosing period. Of these 28 patients, 26 were followed in the open-label study and did not require subsequent doses of alefacept for a median of 10 months (range, 6–18 months). The selective immunomodulatory effect of alefacept on key mediators of psoriasis may contribute to the lasting remission, lack of disease rebound, and favorable safety profile observed in phase II studies.

309

Subcutaneous Injection of MEDI-507, an Anti-T-Cell Monoclonal Antibody, for the Treatment of Psoriasis: Phase I Results

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MEDI-507 is a humanized IgG1 monoclonal antibody that binds to the CD2 receptor of T-cells and natural killer cells. Abnormal T-cell activity has been shown to be important in the development of psoriasis. It has been observed *in vitro* that MEDI-507 has the potential to selectively suppress the function of the immune system. Dosing by subcutaneous (SQ) injection is considered preferable to intravenous (IV) infusion for chronic treatment of psoriasis. MEDI-507 in the range of 0.1–7 mg has been evaluated in a Phase I/II, open-label, dose-escalation study involving 12 weekly SQ injections ($n = 39$). Entry criteria required 10% body surface area psoriasis involvement and a prior treatment washout period. Clinical response is assessed using the Psoriasis Area and Severity Index (PASI). The majority of adverse events reported to date have been transient and judged mild in severity with the most common adverse events including headache, injection site reaction (mild erythema), decreased lymphocyte count, and asymptomatic changes in vital signs during the postdose observation period. Following multiple injections, reductions in PASI are observed at all dose levels although more clinically significant disease improvement is seen at the higher dose levels. Changes in absolute lymphocyte counts are less pronounced following weekly SQ dosing when compared to comparable doses of MEDI-507 given by weekly IV infusion. Preliminary data indicate that SQ MEDI-507 suppresses disease activity and is well tolerated with minimal site of injection reactions. A Phase II program has been initiated in Europe.

310

Phase I Results of Intravenous MEDI-507, an Anti-T-Cell Monoclonal Antibody, for the Treatment of Psoriasis

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MEDI-507 is a humanized IgG1 monoclonal antibody that binds to the CD2 receptor found on the surface of T-cells and natural killer cells. It has been observed through *in vitro* model systems that MEDI-507 has the potential to selectively suppress the function of the immune system. Abnormal T-cell activity has been shown to be important in the development of psoriasis, therefore modulation of T-cell activities may provide an effective treatment for psoriasis. Intravenous (IV) administration of MEDI-507 has been evaluated in a Phase I, open-label, single-dose IV safety study ($n = 14$) and a Phase I/II, open-label, dose-escalation study involving 8 weekly IV infusions ($n = 26$). Entry criteria required 10% body surface area psoriasis involvement and a prior treatment washout period. MEDI-507 doses in the range of 0.4–40 μg per kg were evaluated. Clinical response was assessed using the Psoriasis Area and Severity Index (PASI). The majority of adverse events reported were transient and judged mild to moderate in severity. The most common related adverse events include chills, headache, and decreased lymphocyte count. Dose-dependent reductions in target lymphocyte populations were observed. Clinically significant disease response was observed at dose levels at or above 1.2 μg per kg MEDI-507. The 40 μg per kg dose resulted in the highest peak serum levels and the highest disease activity. Of the 6 volunteers treated at this dose level, 50% experienced a greater than 75% improvement in PASI as compared to entry baseline. Preliminary data indicate that multiple IV infusions of MEDI-507 are well tolerated and that clinically meaningful improvements in psoriasis have been observed. A Phase II program has begun in North America.

311

A Multiparameter Flow Cytometric Analysis of the Effect of Bexarotene on the Epidermis of the Psoriatic Lesion

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Recently, a new drug bexarotene (Targretin®) was investigated in a large multicentre trial for its possibility as an antipsoriatic treatment. Bexarotene is a novel synthetic retinoid X receptor (RXR)-selective retinoid. Retinoids are known to act as modulators of epidermal growth and differentiation. In psoriatic keratinocytes, they induce normal differentiation and suppress proliferation. Therefore, the aim of the present study was to analyse these processes in epidermal single cell suspensions derived from lesions of psoriatic patients treated with various doses of bexarotene. 34 patients with moderate to severe plaque psoriasis participated in this study and were assigned in sequence to four different dose panels: 0.5, 1, 2, and 3 mg per kg once daily for a period of 12 weeks. Before and after 12 weeks of treatment, punch biopsies were taken from lesional skin and epidermal single cell suspensions were prepared using an optimised thermolysin-protocol. To establish a comparison of clinical and flow cytometric data, a sum of scores was determined for each biopsy-site, based on a four-point scale for erythema, induration and desquamation. An improved multiparameter flow cytometric assay was used which enabled simultaneous assessment of epidermal proliferation, various aspects of differentiation and epidermal inflammation. The following parameters were measured simultaneously: DNA-distribution (DAPI), cell size, keratin 10 (RKSE 60), keratin 6 (LHK6B), and vimentin expression (V9). Isotype specific secondary antibodies conjugated with phycoerythrin and fluorescein isothiocyanate were used as the second step in the staining procedure. Differences in cell size and intermediate filament expression enabled us to distinguish inflammation cells from keratinocytes. After 12 weeks of treatment, the subpopulation of keratin 10 only expressing cells showed an average increase of 89.9% of the value before treatment. Additionally, the subpopulation of keratin 6 only expressing cells showed an average decrease of 32,8%. The subpopulation of keratin 6 and 10 coexpressing cells showed a slight increase of 17,8%. In conclusion, treatment with bexarotene results in a marked decrease of epidermal hyperproliferation and normalisation of keratinisation in moderate to severe plaque psoriasis.

312

Evaluation of a New X-Ray Fluorescent Analysis Technique for the Creation of a Nordic Hair Database. Elemental Distributions within the Root and the Virgin Segment of Hair Fibres

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A new, non destructive X-ray fluorescence technique for quantitative estimation of elemental content in biological tissues has been developed. Technical and instrumental characteristics of the ITRAX X-ray spectrometer have been evaluated in relation to the properties of biological samples, i.e. human hair fibres. Thus, attenuation variations of the fluorescent X-rays in the hair bulk mass was demonstrated at analysis of sulfur, calcium and zinc in a part near the root of one hair fiber with elliptical cross section. By rotation of the hair fiber and successive analyzes made of the same part of the hair fiber the results showed that concentrations of elements varied as functions of the diameter of the analyzed hair volume. Other sources of errors are also discussed. The ITRAX instrument allows for precise, fast, nondestructive, simultaneous, quantitative recording of the detected elements and trace elements down to levels of 1 p.p.m. (μg per g). The instrument was used for assessment of normal values of physiologically important elements present in hair in a cohort of normal, healthy Swedish, Caucasian individuals. The database erected from data retrieved from a conceivably homogenous ethnic set of individuals represents to our knowledge the first of its kind.

313

Spectral Anlysis of Malignant Melanoma

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Noninvasive dignostic methods to determine the diagnosis of malignant melanoma become more and more important. Recent studies report on spectral differences between normal skin, naevocytic naevi and malignant melanoma (Marcchesini et al. 1991, 1992, 1995; Wallace et al. 2000). The method of reflection-spectroscopy and image analysis by Fourier interferometer (FIRS and FIBA, respectively) are two methods to measure and put into image the spectrum of optic reflection of the skin. The intention of the study was to investigate spectral differences between normal skin, benign pigmented lesions (BPL) and malignant melanoma (MM) to aim for a possible new method for early diagnosis of MM. Until now, 13 patients with MM have been investigated. Due to technical reasons, only 9 patients could be evaluated. 5 (2f/3m) presented with superficial spreading melanoma (SSM), 3 (1f/2m) with nodular malignant melanoma (NMM) and one with lentiga maligna melanoma (LMM). Normal surrounding skin of the patients and several BPL served as a control group. A Xenon lamp with the light spectrum of 230–1300 nm was used as the light source for FIRS. Reflection images were taken by an attached CCD camera, clculation and imaging was done with FIBA computer program analysis (Mosenheuer, 1993). The outcome was presented by diagrams showing the reflection spectrum (amplitudes) and relative reflection spectrum over the wavelength of 400–1200 nm. Reflection spectrums of BPL and MM demonstrated characteristic curves. Up to 600 nm, BPL showed less reflection than the surrounding skin. Concerning the wavelength of hemoglobin absorption, the reflection of BPL was similar to normal skin, i.e. no enhanced blood supply could be seen. At 600 nm, reflection of BPL was even higher than the surrounding skin, resulting in a characteristic bend of the curves. Reflection curves of MM differed significantly from BPL. Within the absorption spectrum of hemoglobin, MM showed a higher reflection due to higher vascularisation. At 600 nm MM didn't demonstrate the characteristic bend as seen in BPL. In summary, MM demonstrate chracteristic reflection curves in FIRS and FIBA due to structural and vasculary alterations resulting in changed optical qualities of the tissue. FIRS together with FIBA seems to be a promising noninvasive method for diagnosis of malignant melanoma. Since it is still innovative and experimental, further investigations with a higher number of patients are needed.

314

Detection of Circulating Malignant Melanoma Cells in Peripheral Blood by Quantitative RT-PCR Assay

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Early identification of metastases in patients with melanoma is critical. We developed a nested RT-PCR assay for detection of tyrosinase (tyr) mRNA by real-time fluorescent RT-PCR. Consistent detection of tyr negative RNA spiked with tyr positive RNA from malignant melanoma cells was achieved. Total RNA was extracted from blood of 17 patients with stage IB-IV melanoma. All 4 stage IV patients tested positive while both stage IB patients tested negative. One of 9 patients with lymph node metastases identified only by tyr RT-PCR on node tissue was positive, consistent with rates of disease progression. One stage IV patient with a positive assay prior to and during treatment converted to a negative result at the time of radiographical remission. We next developed a novel quantitative RT-PCR assay to detect tyr mRNA in blood using our primer pair and flourescent probe. Using spiked RNA, a sensitivity of 100 cells per ml of blood was achieved. We are currently applying this quantitative assay clinically. In summary, we developed a nested PCR assay with potential clinical utility, and a novel quantitative tyr mRNA assay for further testing. Our goal is to develop a blood test that will improve the initial staging of melanoma, allow for better monitoring of disease progression, and ultimately result in more timely and effective therapy.

315

Human Papillomavirus (HPV)-DNA Sequences in Melanoma Specimens

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Although the etiology of melanoma is still unknown, UV radiation from sun exposure, in particular in people with light hair and skin and with numerous nevi, seem to play an important role. Viruses, especially Human Papillomavirus (HPV), have been implicated in the pathogenesis of cancer, including nonmelanoma skin cancers. In our study, we investigated the possible presence of HPV-DNA sequences in melanoma biopsy specimens. Two polymerase chain reactions (PCR) were used for the detection of different genotypes of HPV on 4 melanoma biopsy specimens, paraffin included, obtained from 4 patients with melanoma. We used two sets of consensus primers located within the L1 region of HPV genome, MY11/MY09 and GP5+/GP6+. The GP primers can be used in the nested PCR following amplification with the MY primers. The amplification products of both PCRs were hybridized with two sets of type-specific biotinylated probes for HPV types 16, 18, 31, 33, 35, 39, 45, 52 and 58. The hybrids were captured on streptavidin-coated microtitre plates and detected by ELISA. No typeable specimens were evaluated by DNA electrophoresis. The best results were obtained when the GP primer sets were used. In fact any samples were positive only with GP primers. The possible reasons for that are smaller size of the PCR product (≈140 bp GP product against ≈450 bp MY product) and that the GP primers do not contain degenerated bases. Furthermore the use of MY/GP nested PCR increased significantly the positivity rate of HPV-DNA detection and should be used for samples with a low copy number of HPV-DNA. The use of the two sets of primers individually in PCR or together in a nested PCR has permitted to detect DNA sequences of HPV 18 and 31 in one patient and HPV-16 in an other melanoma patient. These HPV-genotypes are considered at high risk for cancer development in nonmelanoma skin cancer. The possible pathogenetic role of HPV in melanoma is now under discussion and needs further investigations.

316

Clinical Efficiency of Cytotoxic T Cells as Adjuvant Treatment in Melanoma

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Specific cytotoxic T lymphocytes of melanoma antigens (TIL) are considered as crucial in the antitumor response. However, until now a direct correlation between specific T cytotoxic activity against tumor cells and clinical response has never been demonstrated in melanoma. This is the aim of our study. 88 patients in stage III (ASLL) with clinical lymph node have been included in an adjuvant randomized open study after positive lymph node resection. One arm ($n = 44$) received IL2 alone during 2 months, 6 weeks after the lymph node resection and the other arm ($n = 44$) received TIL and IL2 in the same condition. Moreover, among the TIL arm, the percentage of specific cytotoxic T lymphocytes has been determined in 26 patients by studying the intracytoplasmic production of γ interferon by the TIL incubated with autologous melanoma cell line. With a median follow up of 3 years, this study shows a significant decrease of relapse (73% without relapse in TIL arm vs. 44% in IL2 arm $p = 0.019$) and an increase of survival in patients treated with TIL with only one invaded lymph node ($p = 0.026$). The percentage of specific TIL injected varied between 0.3% and 10%. The injection of specific TIL was significantly associated with a decrease of relapse and an increase of survival ($p = 0.02$) compared with patients injected with non specific TIL. In conclusion, this study demonstrates a clinical benefit of TIL used as adjuvant therapy in melanoma stage III and for the first time a correlation between relapse and overall survival and the injection of specific cytotoxic T cells. At the moment, we begin another study injecting only clonal cytotoxic T cells against MELAN-A/MART-1 antigen in patients stage IV.

317

An Oral 5-Lipoxygenase Inhibitor Reduces Inflammatory Lesions, and Total and Pro-Inflammatory Sebum Lipids in Acne Vulgaris

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Despite the wide consent that P. acnes is a major causative factor for acne, there is evidence which calls in question bacterial involvement in the initiation of acne lesions. In addition, antibiotics that are successfully used in the treatment of inflammatory acne, also exhibit an anti-inflammatory activity. However, anti-inflammatory compounds have been barely used in acne treatment. Therefore, a clinical proof of principle (1C) study was conducted to treat inflammatory acne vulgaris with an oral selective 5-lipoxygenase inhibitor, aiming to control molecular steps of inflammation. Ten patients with acne papulopustulosa (m:f 6 : 4, aged 19 ± 5 years) were treated with (±)-1-(1-benzo[b]thien-2-ylethyl)-1-hydroxyurea 4 × 600 mg per day p.o. for 3 months. Documentation was performed at baseline, and weeks 2, 4, 8, 12, and 14. The acne severity index continuously decreased in a time-dependent manner to 41 ± 28% of the initial score at week 12. This was due to a 71 ± 24% decrease of inflammatory lesions, while comedones did not respond. No adverse events were registered during and after treatment. Interestingly, total sebum lipids significantly decreased and the squalene/cholesterol ratio, free fatty acids and lipoperoxides were markedly diminished in patients' sebum under treatment, whereas the magnitude of clinical improvement strongly correlated with the reduction of total sebum lipids and free fatty acids. In contrast, the levels of leukotriene B_4 in blood and of the casual skin surface lipids were not affected. All parameters studied remained practically unchanged during the 2-week post-treatment observation period. In conclusion, inflammatory acne significantly responded to an oral 5-lipoxygenase inhibitor. Moreover, systemic inhibition of arachidonic acid metabolism reduced total and pro-inflammatory sebum lipids; the latter probably being responsible for the development of inflammatory acne lesions.

318

Testosteron Level in Female Acne Patients

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Pathological process in acne is in sebaceous follicle. Sebaceous glands are target organ for human androgens. In female, the role of androgens is weak, but if these influences are stronger, side-effects are more obvious. Testosterone is the most important regulator of sebaceous function. Reports from literature are contradictory, notifying testosterone level in acne patient in compare with healthy individuals. We have compared examining group of acne patient (105) with healthy control (30). Female acne patient has been classified according two main criteria: age and clinical findings. Testosterone was detected by RIA method twice in interval of two months, with J125 (Farmos Diagnostic Oulunsalo Finland). According to our investigation total testosterone level (arithmetical medium) is greater in experimental group of patients. Total testosterone level is in correlation with severity of clinical picture in all age groups. Total testosterone level is significantly higher in patients with acne then in healthy controls. This is especially case in more difficult clinical pictures as acne nodulo-cystica and acne conglobata.

319

Cutaneous Sensory Nerve Fibers are Decreased in Number Following Peripheral and Central Nerve Damage

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Two dermatological patients, dysplaying peripheral and central nerve damage, respectively, are described. Cutaneous nerve fibers in both patients were studied in skin biopsies taken from neuropathic areas and from the contra-lateral side, immunocytochemistry being applied to a pan-neuronal marker, a protein gene-product (PGP 9.5). One of the patients, suffering from compression of the ulnar nerve, had dishidrotic eczema of the hands that was absent on areas of skin that were neuropathic. The cutaneous innervation (most of which being sensory) was reduced by 50% in the neuropathic area as compared with the contra-lateral side. The other patient had unilateral pruritus on the parethic side following a stroke. The cutaneous innervation of that side was reduced by 80% as compared with the other side. It seems that peripheral sensory innervation is a prerequisite for inflammation while spontaneous itching may emanate from a central nervous system disorder such as a stroke and continue on in partly denervated skin.

320

Pemphigus Sebaceous Assosiated with Scleroderma Diffusa

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We observed a woman of 50 with pemphigus sebaceous and scleroderma diffusa. During 10 years the patient was treated by glucocorticosteroids and metotrexate in Reumatological Center. Dermatological manifestations appeared after contact with toxic chemical products. The patient was hospitalized in dermatological clinic. Clinical signs were revealed through multiplied blisters on the skin of the trunk, face, hands and legs. On the legs were sclerotic and atrophic changes. The acantholytic cells of Tsank were found out. The state of the patients was hard. The patient received glucocorticosteroids and her state turned out to be better. This case demonstrated the rare association of two autoimmune diseases in the patient, who was treated for a long time by immunesupresivetherapy.

321

Induction of Partial Tumor Regression in Cutaneous T Cell Lymphoma by Anti-Idiotype Vaccination

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Peptides derived from the complementarity determining region (CDR)-3 of the tumor-specific T cell receptor (TCR) are known to be recognized by autologous HLA class-I restricted CD8+ T cells. The present study aimed the determination of immunogenic idiotype sequences and their capacities to induce clinical and immunological responses in CTCL patients. After sequencing the tumor-specific TCRβ of four patients with different HLA types, epitopes were predicted that match both, the proteasome cleavage specificity and the binding requirements of the respective HLA molecules using an own algorithm for the former and the SYFPEITHI database for the latter. CD8+ T cells specific for the nonapeptides with the highest predictive scores were demonstrated in all patients by intracellular IFNγ staining of peptide activated cells. In one patient with tumor stage Mycosis fungoides, vaccination with these nonapeptides together with helper T cells inducing antigens led to partial remission of the tumor nodules. Each vaccination boostered enhanced frequencies of peptide-specific CD8+ T cells in the peripheral blood. Since tumor-specific TCRβ DNA but not mRNA was detected in the remaining tumors, resistance of the neoplastic cells in this case might be mediated by down-regulation or loss of expression of the tumor-specific TCR.

322

Patients with Chronic Graft vs. Host Disease have Amplified Circulating Clonal T Cell Populations: Evidence for a Possible Correlation with Response to Photopheresis

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Chronic graft vs. host disease (cGVHD) is a major complication of bone marrow transplantation (BMT) and is responsive to photopheresis, a treatment that induces an anticlonotypic immune response and has proven to be effective in cutaneous T cell lymphomas with circulating clonal T cells. We screened blood samples of 23 patients after HLA-matched allogeneic bone marrow transplantation (allo-BMT), 10 without cGVHD and 13 with extensive cGVHD, for clonal T-cell receptor γ (TCRγ) gene rearrangements using fluorescent-based polymerase chain reaction (PCR) and automated high-resolution capillary electrophoresis. Amplified clonal populations of T cells with unique TCRγ gene rearrangements were found in 6 of 10 (60%) allo-BMT patients without cGVHD and 11 of 13 (84.6%) allo-BMT patients with cGVHD, as compared to 0 of 10 (0%) healthy controls. Eight patients with cGVHD were treated by photopheresis, and the presence of amplified clonal populations of T cells was found to be associated with a cutaneous response to photopheresis, as 6 of 6 (100%) clone positive vs. 0 of 2 (0%) clone negative patients experienced a clinically significant cutaneous response to treatment. Our findings suggest that a high proportion of patients with cGVHD have detectable expanded clonal T cell populations in their peripheral blood, and such patients may be more likely to respond to treatment by photopheresis.

323

Induction of Granulation Tissue Formation in Chronic Wounds by Hyalofill-F® (a Derivative of Hyaluronan)

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Background Hyalofill-F® is a nonwoven benzyl esterified hyaluronic acid derivative. When applied to a wound, it hydrolyses and provides a hyaluronan-rich environment. Hyaluronan, a linear polymer of glucuronic acid *N*-acetylglucosamine disaccharide, has been shown to be associated with multiple biological events in wound healing and promotes tissue repair and angiogenesis in experimental models. Several case reports and descriptive studies on the clinical use of hyaluronan have been published, but controlled clinical trials are scarce. The purpose of this study was to find clinical evidence for the hypothesis that hyaluronan derivatives induce granulation tissue formation. Because hyaluronic acid gauzes form a gel after contact with wound fluid, another gel (IntraSite gel) was used as control treatment.

Methods In 10 consecutive patients with large nonhealing ulcers, caused by venous insufficiency (8) or vasculitis (2), part of the ulcers was treated with hyaluronic acid (Hyalofill-F), while the other part was treated with IntraSite gel. Wound size, time until a red granulating wound bed was achieved, total wound healing time, and number of required grafting procedures were recorded.

Results Compared to control, time until grafting was reduced with 7 days (29%, $p = 0.004$), total wound healing time was reduced with 19 days (31%, $p = 0.0003$). The hyaluronic acid-treated ulcers required 6 grafting procedures, vs. 11 in the control group.

Conclusion The advantage of this *intrapatient or intraindividual comparative study design* is that important confounding variables, such as ulcer duration, etiology, age, nutritional state, glucose levels, general condition, smoking, hypertension, and compliance can be eliminated. By using this special study design, we were able to conclude that Hyalofill-F®, a hyaluronan derivative, is able to stimulate granulation tissue formation and wound healing. Based on this study, we recommend that hyaluronan should be tried in yellow necrotic ulcers that fail to respond to conventional treatment.

324

Image Analysis Techniques in Evaluation of the Healing Process in the Course of Chronic Leg Ulcers

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Image analysis techniques have a long-standing tradition in medicine. They are regarded to be of considerable help in precise, quick, objective and automated characteristics of different objects. Nowadays, computed image analysis techniques are used in a vast majority of morphometric studies (e.g. for individual cell description). They are also introduced in evaluation of some skin lesion extensiveness. The aim of our study was to estimate healing process progress in the course of chronic leg ulcers on the basis of computed image analysis technique measurements of ulcer area. The study was performed on series of photographs obtained from 22 patients with chronic leg ulcers in the course of intensive inpatient and outpatient treatment. This material was subsequently analysed by image analysis procedures performed using a number of subroutines written in Delphi developed at the Institute of Electronics, Technical University of Lódz. The analysed images were scanned using HP 3c-colour scanner in true-colour (24 bits per pixel) format, with size 512×512 pixels each. Next, image histogram stretching procedure was performed to improve image contrast. Subsequently, thresholding was performed to visualize leg ulcer lesions. Then, geometrical features of the objects such as edge and area of the leg ulcers were calculated. However, at the beginning of the study we had some problems with estimation the edge and subsequently area of the ulcers which were tightly covered with purulent exudates. Overall, the employed method allowed for evaluation of leg ulcer healing process on the basis of their area decrease in an objective and precise way. Demonstration of such objective results was also appreciated by the patients, who were predominantly above 60 years of age. This method exerted a surprisingly good impact on psychological approach of the patients to therapy.

325

Psoriasis is Highly Associated with Increased Risk for Fatal Cardiovascular Disease

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Conflicting data exist regarding the risk for cardiovascular disease in psoriasis patients.

Methods To assess the risk for fatal cardiovascular disease such as myocardial infarction and stroke in psoriasis patients, we have performed a nation-wide large-scale epidemiological study using population based national registries, the Swedish Causes of Death registry and the Swedish Discharge registry. The latter started in 1964 and is built up on a county-based basis encompassing an increasing number of counties over time, in 1964–20% and in 1987–100%. The registry comprises all episodes of in-patient care in Sweden identifying in this study a total of 10.667 patients, 5.638 males and 5.029 females, who were followed for 15 years or more. All patients had psoriasis as first diagnosis. Patients with psoriasis as secondary diagnosis or who reported an episode of cardiovascular event prior to being diagnosed with psoriasis were excluded (appr 10.000). Risks are presented as standard mortality ratio (SMR), i.e. the ratio between the observed and the expected number of deaths, and 95% confidence intervals (95%CI).

Results Overall, 2255 deaths caused by cardiovascular events, were observed in psoriasis patients compared to the expected 1257. The risk for cardiovascular death increased with increasing number of admissions to the hospital. The youngest age group, 20–40 years, had the highest risk, SMR 2.85; (95% CI 3.75–4.95, the latter patients being admitted at least three times for in-patient care. At age 40–60 the corresponding risk was 1.91; (95% CI 2.13–2.31).

Conclusion Psoriasis patients who are hospitilized with psoriasis as first diagnosis have overall a two-fold increased risk for fatal cardiovascular disease compared to the general population. The risk increases with decreasing age and increasing number of hospitalizations suggesting a correlation between severity of psoriasis and risk for cardiovascular death. Whether cardiovascular morbidity is a consequence of systemic inflammation or reflects deranged blood lipids,smoking habits or other remains to be elucidated.

326

Relevance of Immunosuppressive Load and Other Risk Factors in the Development of Squamous Cell Carcinoma (SCC) and Basal Cell Carcinoma (BCC) in Heart Transplant (HT) Recipients

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HT recipients show an increased incidence of cutaneous malignancies, with a reversal in SCC/BCC ratio compared with the general population. What induces such reversal has not yet been fully clarified. We studied 360 HT recipients (302 male, mean age at HT 49 ± 16 years); mean post-HT follow-up was 5.5 years (range: 1 month – 14 years). Each patient underwent regular dermatological follow-up. Multivariate analysis (Cox regression) included: age at HT, sex, warts and solar keratosis, skin type, occupational sunlight exposure, number of treated acute rejection episodes during the 1st post-HT year, 1st year post-HT cumulative dosage of i.v. boluses of methylprednisolone and cumulative azathioprine, cyclosporine A and prednisone dosage at 3 months, 6 months, 1 years, 2 years, 3 years post-HT. In order to evaluate the global immunosuppressive load, we also included the weighted linear combination (WLC) of the cumulative azathioprine, cyclosporine A and prednisone dosage for each patient at 3 months, 6 months, 1 years, 2 years and 3 years post-HT. Fifty-seven HT patients (15.8%) developed a total of 131 nonmelanoma skin cancers after HT (90 SCC; 41 BCC; SCC/BCC ratio 2.19 : 1). The cumulative incidence was 9% (95% CI, 5%-13%) and 20% (95% CI, 10%-30%) for SCCs and 8% (95% CI, 4%-12%) and 14% (95% CI, 6%-22%) for BCCs after 5 and 10 years, respectively. Age at HT > 49 years ($p = 0.02$; RR = 4.4), occupational sunlight exposure > 30000 h ($p = 0.01$; RR = 2.7), solar keratosis ($p < 0.0001$; RR = 6.1), WLC of the cumulative azathioprine, cyclosporine A and prednisone dosage at 3 years post HT ($p = 0.05$; RR = 2.4) were risk factors for SCC. Age at HT > 49 years ($p = 0.02$; RR = 4.4) and skin type 2 ($p = 0.0003$; RR = 6.4) were risk factors for BCC. Our study confirms the well known importance of cumulative sunlight exposure for the development of SCC and the association between skin type and BCC; it furthermore suggests that the risk of SCC, but not of BCC, in HT recipients is related to a higher level of immunosuppression. This could tentatively explain the reversal of the SCC/BCC ratio on the basis of an escape from the immunosurveillance mechanism more relevant for the highly immunogenic SCC than for the less immunogenic BCC.

327

Transforming and Biochemical Properties of the HPV77 E7 Protein, a Novel Papillomavirus Isolated from a Renal Transplant Recipient

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Renal transplant recipients (RTRs) suffer from a 50–100 fold increased risk of developing squamous cell carcinomas (SCCs) which may be linked to infection with specific human papillomavirus (HPV) types. A broad spectrum of HPV types have been found in these lesions including many novel types. We have investigated the transforming potential of one of these, HPV77. Structure-function analysis was used to investigate the HPV77 E7 protein in terms of its ability to transform immortalised rodent fibroblasts and bind the Rb tumour supressor protein *in vitro*, and compared this to the known transforming ability and binding capacity of HPV16 E7. We show that the HPV77 E7 protein transforms immortalised rodent fibroblasts, although at slightly reduced levels to HPV16 E7 and in contrast to the E7 proteins of two other cutaneous HPV types, HPV5 and HPV10. Motifs in HPV77 E7 revealed the presence of pRb binding and casein kinase II (CKII) phosphorylation sites. Using recombinant HPV GST fusion proteins produced in bacteria, we show that HPV77 E7 is capable of binding pRb *in vitro* produced in a transcription/translation coupled reaction. Furthermore we demonstrate that the HPV77 E7 protein is a good substrate for CKII phosphorylation. In contrast to HPV16 E7, site-directed mutagenesis of the two serine residues of the HPV77 E7 CKII phosphorylation site to either alanine or aspartate, to mimic the unphosphorylated or phosphorylated forms of HPV77 E7, respectively, abolished its transforming ability. These results demonstrate that HPV77 E7 is a transforming protein that shares homology with HPV16 E7 in specific regions known to be important for its transformation function. However, important differences in the regulation of this transforming ability are in evidence between HPV16 E7 and HPV77 E7 in terms of the effects of CKII phosphorylation.

328

Factors Affecting the Expression of Lipases by the Skin Commensal, Staphylococcus Epidermidis

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Staphylococcus epidermidis is the dominant staphylococcal strain isolated from human skin and although historically considered to be a nonpathogenic commensal, has emerged as an important cause of nosocomial infections. *Staphylococcus epidermidis* predominantly inhabits regions of the skin which are rich in sebaceous lipid, such as the face, upper chest and back. Therefore, as part of research to identify factors important for colonisation of normal human skin, the lipolytic activity of *S. epidermidis* was examined. *Staphylococcus epidermidis* produces two different lipolytic enzymes, GehC and GehD. To study the differences between these enzymes, *in vitro* reporter gene fusions for each of the lipases were constructed and the effects of a number of factors on gene expression were tested. As is common for many staphylococcal exoproteins, both lipases were found to be produced at the postexponential phase of growth. Furthermore, induction of exponential phase expression upon addition of stationary phase cell-free supernatant suggested they may be regulated by a cell density-sensing system. Lipase expression was increased upon addition of ethanol or the sebaceous triglyceride, triolein to cultures while being reduced in the presence of oleic acid. Expression of both lipases was also strongly repressed by addition of glucose. Although the genes are not located together as an operon, these results indicate a co-ordinated method of regulation for *S. epidermidis* lipases and suggest that they may be important for colonisation of staphylococci on human skin.

329

Absence of Anti-Human Herpesvirus 8 (HHV-8) Antibody in 32 Japanese Hemophiliacs with the Advanced HIV Infection

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It is well known that the incidence of Kaposi's sarcoma (KS) is high, 20–45%, among HIV-positive homosexual men infected through sexual contact. Their seroprevalence of HHV-8 has been reported as 90%. In contrast, no KS were seen in any of the 53 Japanese HIV-positive hemophiliacs during more than the 13-year follow-up period after seroconversion to HIV. To clarify the seroprevalence of HHV-8 in this group, we used a sensitive mixed-antigen ELISA test using 4 recombinant proteins encoded by ORF 59, 65, 73 and K8.1. Sera were available from 32 out of 53 patients. The examination demonstrated that none of the serum samples from the 32 HIV-positive hemophiliacs contained anti-HHV-8 antibodies. The seropositivity rate of HHV-8 was no more than that in the general Japanese population (1.4%). This study showed that the prevalence of KS and seroprevalence of HHV-8 were no higher in Japanese HIV-positive hemophiliacs exposed to HIV through blood product. Our data supports the previous notion that HHV-8 infection is strongly related to the development of KS in HIV infected individuals.

330

Association Between Seasonal UV Exposure and the Incidence of Chickenpox and Shingles

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UV radiation is recognised to suppress immune responses, and cell-mediated immunity is important in the control of zoster virus during chickenpox and shingles and in the latent period in-between. The aim of the present study was to determine whether the incidence of chickenpox and shingles occurring in a defined area of Lodz correlated with solar UV irradiance. The irradiance was measured daily in Warsaw, about 120 km north-east of Lodz, for the years 1999 and 2000 and was expressed as minimum erythemal dose (MED) where one MED was defined as 21 mJ per cm2 effective radiation. The monthly irradiance ranged from about 10 in December to about 400 MEDs in June/July. The number of cases of chickenpox and shingles occurring in each month over the same two year period was also recorded. For chickenpox, they totalled 14 865 (range 120–1123 per month) and, for shingles, 585 (349 female and 236 male, range 13–46 per month). Seasonal variation was assessed by 'cosina' analysis in which the first terms in the Fourier series were fitted in a Poisson regression model for numbers of cases of chickenpox/shingles and a linear regression model for UV irradiance. Rank correlations between the monthly UV and the clinical cases were computed. The seasonality of UV was highly statistically significant ($p < 0.001$). Significant evidence of seasonal variation was found for total shingles ($p = 0.04$) and for shingles in males ($p = 0.02$) with the lowest number of cases in the winter. For chickenpox, there was highly statistically significant evidence of seasonality with the maximum number of cases in March and the minimum in August/September. There were quite large, although nonsignificant, negative correlations of solar UV/day and shingles cases per day with chickenpox cases per day. Thus the pattern of seasonality with regard to the incidence of these two infections was quite different. The change in chickenpox numbers with season is probably due to the ease of spread of the virus by indoor living in the winter months. For shingles, it is tempting to speculate that the increase in solar UV in the summer could down-regulate immunity, hence contributing to the reactivation of the virus and the increased incidence.