P001

Aachen Atopy Array: A novel allergen chip technique for the analysis of serum IgE antibodies to recombinant latex allergens

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Allergy to latex takes a special position within type-I-allergies. High-risk-patients are health-care workers, patients with atopic disposition and patients who undergo frequent surgical procedures. Routine in vitro and in vivo tests for latex allergy uses no-standardized allergen extracts and therefore vary in sensitivity and specifity. To date, 12 latex proteins have received designation as allergens (Hev 1 to Hev b 12) and 8 of them are cloned as recombinant allergens. We investigated 7 of them using a novel microarray-based method, the Aachen Atopy Array (r Hev b 3, r Hev b 5-10). In this test, a computer-assisted qualitative and quantitative analysis of interacting human IgE antibodies with an array of recombinant allergens was performed incubating only 5 µl of patient serum on a solid-phase chip. In a large group children and adults with atopy we could show that results of Aachen Atopy Array correlate excellent with skin prick tests and established enzyme-immunoassays using inhalant and nutritive allergens. In 7 adult patients we found high and medium levels of specific IgE against recombinant latex allergens, especially r Hev 6, 8 and 5. These findings were confirmed by established enzyme-immunoassays showing similar levels of IgE expression. The Aachen Atopy Array is a novel and fast immunoassay using recombinant molecules and allows the identification of specific IgE-antibodies to nutritive and inhalant allergens, among latex proteins. This test shows excellent correlation with established in vivo and in vitro diagnostic tools. Additionally, this technique may be used to improve the uncertain diagnostic of latex allergy especially in occupational and environmental medicine.

P002

Inhibition of IgE synthesis by docosahexaenoic acid (DHA) in different allergy models

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The increase of atopic diseases including atopic dermatitis, allergic rhinitis and asthma has been linked to environmental factors such as an altered consumption of polyunsaturated fatty acids (PUFA). These inflammatory diseases are associated with an IgE response to common environmental allergens. Several studies indicated that n-3 PUFAs, e.g. DHA, have immunomodulatory activities. In the present study we analysed the anti-inflammatory effect of DHA on IgE synthesis in vitro and in vivo.DHA inhibited significantly the CD40+IL-4-mediated IgE production from PBMC, but not from B cells, from donors with serum IgE levels below 1000 pg/ml in a dose dependent manner (10-5M: -79%; 10-7M: -47%; n=6). By contrast, in vitro production of IgA and IgM was not significantly influenced by DHA after CD40+IL-4 stimulation, and IgG synthesis was slightly but significantly reduced (10-5M: -32%; 10-7M: -20%; n=6). Moreover, the presence of DHA did not influence IgE synthesis affecting cytokines IFNgamma, IL-2, IL-6 and TNFalpha in vitro and DHA also did not alter the CD40+IL-4-mediated proliferation of PBMC and B cells. Data from DHA fed and OVA-sensitized mice revealed a total inhibition of OVA-specific IgE synthesis, whereas the total IgG1 and IgG2 levels were comparable to the control versus DHA group (n=6). According to this data, DHA supplementation reduced the IL-4 and IFNgamma production in OVA-sensitized mice. Taken together, we show that DHA inhibits IgE production in vitro and in vivo. It suggests a strong immune modulating capacity of DHA or its metabolites in the allergic immune response. As recent epidemiological studies suggest dietary DHA might be of therapeutic interest for the regulation of allergic diseases. The exact mechanisms involved are currently under investigation.

P003

Tuning the immune response by allergen carriers: Th2-polarization induced by pollen associated lipid mediators (PALMs)

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The immune response of atopic individuals against allergens is characterized by an increased release of Th2 cytokines by allergen-specific T-helper cells. However, the way in which the Th cell cytokine profile is matched to the type of invading allergen, and why these profiles sometimes derail and lead to disease, is not well understood. We recently demonstrated that pollen grains not only function as allergen carriers but are also a rich source of bioactive lipid mediators stimulating and attracting cells of the innate immune system. Here we demonstrate that soluble factors released from pollen (APE, aqueous pollen extracts) modulate dendritic cell function that results in an altered T cell polarization. APE dose-dependently inhibited the LPS induced IL-12 production of monocyte derived dendritic cells, while IL-6 release remained unchanged. The ensuing T-cell response of heterologous naive T-cells stimulated by DC matured in the presence of APE resulted in a dramatic shift from a Th1 to a Th2 phenotype as compared to the response obtained with DC matured in the presence of LPS alone. In addition, APE inhibited in DC the LPS induced release of Th1 chemokines such as RANTES and IP10, whereas the release of the Th2 chemokine TARC remained unchanged suggesting a reduced capacity of APE activated DC to attract Th1cells. We also present the chemical analysis of APE with respect to the content of the recently described dinor-isoprostanes, the phyotoprostanes. From the wide array of phytoprostanes identified so far, the cycopentenones PPJ1 (type 1 and 2) exhibted the most prominent inihibitory effect on the LPS induced IL12 production and induced a marked Th2 switch of the allogeneic T cell response. Studies are underway to disect the signal transduction of these lipid mediators. In summary our results demonstrate that pollen associated lipid mediators (PALMs) act as important regulatory signals that modulate DC function in a fashion that favors Th2 polarization.

P004

Inhibition of the IL-4/IL-13 receptor system in a mouse model for atopic dermatitis

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IL-4 and IL-13 are considered as key regulators for the development of atopic disease. However in atopic dermatitis their pathophysiological role and the therapeutic potential of TH2 cytokine antagonists is less understood.
To address this NC/Nga mice were kept under conventional conditions, where they develop spontaneously eczematous skin lesions resembling to human atopic dermatitis. At the age of 4 weeks, mice were either treated with saline or with 10µg of an IL-4/IL-13 inhibitor twice daily for 6 weeks intraperitoneally. The IL-4/IL-13 inhibitor has been shown previously to efficiently inhibit IL-4/IL-13 reactions in vitro and in vivo. Skin lesions were graded macroscopically as summary of individual scores (0-3) for the symptoms itch, oedema, erythema, scaling and erosion. After 12 weeks mice were killed and lesional skin was analysed histologically for acanthosis, hyperkeratosis, and inflammatory infiltrates. IgE levels were detected in the sera by ELISA. In addition, TH1 and TH2 cytokines were measured in the supernatants of in vitro restimulated (IL-2 and anti-CD3) spleen cells.
In saline treated control mice, which were kept under conventional conditions skin lesions were detectable macroscopically at 6 weeks and increased with age. Interestingly skin lesions of mice, which were treated with the IL-4/IL-13 inhibitor, developed significantly faster and stronger macroscopically as well microscopically. In line with the eczema score serum IgE levels were significantly higher in 12 week old mice treated with the IL-4/IL-13 inhibitor, than in 12 week old saline treated mice. Another group of saline treated control mice was kept under specific pathogen free conditions (SPF) and showed no signs of eczema until the age of 12 weeks. In this group serum IgE remained at low levels. All 3 groups of mice showed similar levels of IFN-g in the supernatants of in vitro restimulated spleen cells. In contrast, IL-4, IL-5 and IL-13 were undetectable in SPF mice and increased to similar levels in saline treated and in mice treated with the IL-4/IL-13 inhibitor under conventional conditions.
In conclusion the inhibition of IL-4 and IL-13 seems to increase eczma severity and implies no beneficial therapeutic effect for IL-4/IL-13 antagonists in our mouse model.

P005

Contact hypersensitivity to TNCB but not to DNFB in ß2 microglobulin deficient mice

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It has recently been demonstrated that the dominant effector T cells in contact hypersensitivity (CHS) induced by various haptens or nickel are cytotoxic, IFN-? producing $CD8^+$ Tc1 cells. For $CD4^+$ T cells a regulatory role has been shown. In the current study we have used $CD8^+$ T cell deficient ß2-microglobulin Knockout (ß2m KO) mice which have previously been reported to be unable to mount a CHS response to DNFB in order to reinvestigate a potential effector function of $CD4^+$ T cells. In contrast to DNFB which did not induce CHS, TNCB elicited a robust ear swelling response in ß2m KO mice with intensities and kinetics comparable to C57BL/6 mice. Interestingly, we could show that in the ß2m KO mice DNP-specific, IFN-? producing $CD4^+$ T cells were induced after sensitization, but IFN-? production was not detected after elicitation and in vitro restimulation of these T cells. We are currently analysing the effector mechanisms responsible for $CD4^+$ T cell mediated CHS to TNCB and the factors which determine the striking differences of the two chemically very similar haptens in their capacity to induce CHS. Our data show that, depending on the hapten used, a CHS response can also be mediated by $CD4^+$ T cells in the absence of $CD8^+$ T cells.

P006

Delayed type hypersensitivity reactions to pollen derived factors

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Eczematous reactions to epicutaneous application of aeroallergens are well documented in a subgroup of patients with atopic eczema (AE). Although the pathomechanisms of respiratory atopy are well established, the role of IgE-mediated hypersensitivity in the elicitation and maintenance of eczematous skin lesions in AE is still controversial. The aim of this study was to delineate the effect of pollen grains on cutaneous skin reaction in comparison to a type IV hypersensitivity response: the allergic contact dermatitis to nickel.

Four patients with history of AE, sensitisation to grass and birch pollen and positive APT-reactions were chosen for the study. Pollen grains (grass and birch) were applied in Finn chambers. Patch test reactions were graded according to the guidelines of the European Task Force on Atopic Dermatitis. Biopsies were taken from positive and negative patch test reactions 24, 48 and 72 hours after application and investigated by immunohistochemistry for T cell markers and cytokines.

Histology in both positive pollen and nickel patch tests showed a strong influx of lymphocytes while negative patch test to pollen were devoid of any significant cellular infiltrate. The vast majority of T lymphocytes infiltrating the positive patch test to pollen were characterised as CD3+, CD4+, CD45RO+, with just a small amount of CD8+. Furthermore, a clear percentage of the mononuclear cells were CD25+. This infiltrate was comparable to that induced by nickel even though the kinetic was faster in the pollen patch test showing a cellular infiltrate already after 24 hours. Concerning the cytokine pattern the pollen patch test showed a more pronounced IL5 expression compared to that induced by nickel, while IFN-? was comparable expressed in both cases.

Here we demonstrate that native pollen grains induce eczematous skin reactions in a subgroup of patients with AE. This eczema showed immunohistochemical similarities to delayed type hypersensitivity reactions, but seem to have a faster kinetic and a more Th2 driven pathway than the allergic contact dermatitis to nickel. Since healthy controls did not show clinical reactivity and only a scarce cellular infiltrate, the mechanisms underlying these eczematous reactions seem to be at least in part allergen specific.

P007

Th2 cytokines inhibit bacteria-induced human beta-defensin-2 expression in keratinocytes

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Atopic dermatitis (AD), a chronic inflammatory skin disorder, is often coupled with bacterial and viral skin infections suggesting that antimicrobial defense mechanisms in these patients might be disturbed. It has been shown that atopic dermatitis is often associated with elevated levels of Th2 cytokines like IL-4, IL-10 and IL-13. Recent studies indicated that skin of AD patients contains decreased levels of antimicrobial proteins such as the inducible peptide antibiotic human beta-defensin-2 (hBD-2). Since hBD-2 is strongly induced by mucoid forms of bacteria like *Pseudomonas aeruginosa* (PA), we investigated whether PA-mediated hBD-2 induction in keratinocytes is influenced by Th2 cytokines. To answer this question, we stimulated keratinocytes with PA in the presence of 40 ng/ml IL-4, IL-10 and IL-13 and analyzed hBD-2 expression using Real-time RT-PCR, luciferase gene reporter assays and western-blot analysis.

Stimulation of HaCaT keratinocytes with supernatants of mucoid PA for 16 h resulted in a strong hBD-2 induction which was significantly decreased by co-treatment with Th2 cytokines. IL-4 was found to be the strongest inhibitor of hBD-2 induction followed by IL-13 and IL-10. Quantitative gene expression analysis demonstrated that IL-4 reduced hBD-2 induction by 80-90%, IL-13 reduced hBD-2 induction by 70-80% and IL-10 reduced hBD-2 induction by 20-30%.

Preliminary data indicated that the expression of the skin-derived antimicrobial protein RNase 7 is also influenced by IL-4. Treatment of HaCaT keratinocytes with 40 ng/ml IL-4 for 16 h resulted in approximately 50% reduction in the mRNA level of RNase 7. Co-treatment of HaCaT-cells with IL-4 (40 ng/ml) and PA for 16 h resulted in approximately 50% inhibition of PA-mediated RNase 7 gene induction.

These data indicate that bacteria-mediated induction of antimicrobial proteins in AD might be disturbed as a result of elevated amounts of Th2 cytokines such as IL-4, IL-10 and IL-13. Diminished induction of antimicrobial proteins, in turn, could lead to increased growth of microorganisms leading to an augmented incidence of infections and inflammation in AD patients.

P008

Novel mutation in connexin-26 causes conformational changes and causes KID syndrome

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KID syndrome is a rare autosomal dominant disorder, originally classified as an ichthyosis but rather considered an ectodermal dysplasia because of the complex association of abnormalities. It comprises cutaneous symptoms, auditory abnormalities and ophthalmologic features as well as an increased susceptibility to various infections, defective dentition and other inconstant manifestations. Recently, four different missense mutations in the connexin-26 gene (*Cx26*) were identified in patients with KID syndrome. Here we report a 50 year old patient treated for many years for deafness, who had persistent but quite limited skin lesions, previously neglected. She had erythematous plaques, keratotic and verrucous lesions on her feet, hands, elbows and scalp. Keratitis was diagnosed but did not impair the visual function. When *Cx26* was sequenced, a novel mutation designated as I30N, located in the first transmembrane region of connexin-26, was identified. To verify its putative effect, immunofluorescence studies were performed and showed an altered staining pattern. Secondary structure analysis of connexin-26 revealed that the hydrophobicity of the first transmembrane spanning region including the substitution I30N was strongly reduced. Analysis of polymorphisms within the transmembrane region resulted in no influence on hydrophobicity, whereas the mutations M34T and V37I (previously described in patients with deafness) showed weaker influence. These results sustain that the novel mutation, I30N, induces complex conformational changes of the gap junctions and thereby argues for its pathogenic role.

P009

Capillary blood derived allergy diagnostic in children with bronchial asthma and seasonal allergic rhinitis using CHIP technology

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Background: Diagnosis of type I allergy currently relies on allergen extract-based methods detecting specific IgE antibodies to various inhalant or food allergens. However, allergen extracts may be heterogeneous and vary in allergen composition. We therefore investigated a novel technique of IgE detection based on microarrayed recombinant or natural allergen components. It was the aim of our pilot study to compare the results of this component-based diagnostic tool with two established extract-based methods of type I allergy diagnosis. Methods: In 25 pediatric patients with asthma and seasonal allergic rhinitis allergen-specific IgE antibodies to allergen extracts were routinely detected by an established fluorescence enzyme-immunoassay. A customized allergen microarray was used to assess specific IgE antibodies to 32 recombinant and 32 natural allergen components requiring 5 µl of patient serum. Skin prick testing was performed according to EAACI guidelines. Results: Analysis of capillary and venous blood samples revealed similar results. Statistical analysis focused on the most common inhalant allergens and revealed a statistically significant ($p < 0.05$) association of pooled microarray-data with fluorescence enzyme-immunoassay and skin prick testing results for all analysed allergens.Conclusions: This allergen microarray is a novel and fast immunoassay using 32 recombinant and 32 natural allergen components requiring only small amounts of serum specimen. Especially the use of capillary blood samples offers a new perspective in low invasive pediatric allergy diagnostics. This test shows excellent correlation with established in vivo and in vitro diagnostic tools. Additionally, this technique may be used to improve choice and monitoring of specific immunotherapy by identification of the disease-eliciting allergen molecules.

P010

Stress worsens allergic dermatitis

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Stress is said to exacerbate allergic skin diseases such as atopic dermatitis. Respective research has largely focused on the central activation or cell culture experiments. Interestingly, the stress-mediator substance P was shown to induce interferon-? expression in T-Lymphocytes normally expressing a TH-2-like cytokine pattern in vitro, which is also associated with the chronification of atopic dermatitis. In skin, peptidergic nerve fibres form close contact with immune cells such as mast cells. However, local interactions between the immune and nervous systems, especially under perceived stress, have rarely been reported. Here we show for the first time, that 48hrs after a 24hr sonic stress exposure and induction of allergic dermatitis dermal infiltration e.g. by eosinophils increased significantly in C57BL/6 mice over mice that were either stressed or had allergic dermatitis as well as over untreated controls. Increased infiltration was associated with increased epidermal thickness in stressed mice with allergic dermatitis, and with an increased number of VCAM-immunoreactive blood vessels. At the same time the percentage of degranulated mast cells increased significantly and the number of substance P-immunoreactive peptidergic sensory nerve fibers decreased in the acute allergic dermatitis lesions. Ultrastructural investigation showed unmyelinated peptidergic nerve fibres in a state of deterioration close to degranulating mast cells and eosinophils in the skin of stressed mice with allergic dermatitis, suggesting a decreased number of substance P-immunoreactive nerve fibres due to active release of SP. This may lead to an upregulation of endothelial adhesion molecules and increased infiltration by immunocytes. These data provide first evidence for stress-induced exacerbation of cutaneous allergic diseases such as atopic dermatitis by local interaction of the peripheral nervous system with the immune system.

P011

Antioxidative Activity of Airborne Pollen is Species Specific

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Background: Pollen grains - beyond their function as allergen carriers - are a rich source of bioactive mediators, i.e. linolenic and linoleic acid, and their monohydroxylated derivatives, and flavonoids. Flavonoids deriving mostly from the exine of pollen grains are potent antioxidants. For plants it is known that flavonoids belong to the defense system and are induced after abiotic and biotic stress. This study aimed to show whether pollen grains display antioxidative activity and if so, whether this antioxidative activity is further influenced by abiotic stress - namely the in vitro exposure to pollutants.
Method: In situ exposure experiments of pollen grains were performed imitating natural conditions in a fluidised bed reactor. Birch and grass pollen were exposed to 5 ppm SO2 (30%rh or 50%rh or 70%rh, 16h) and fine particles (urban dust diameter 3µm, 6h). Synthetic air served as control. Aqueous extracts were prepared from exposed pollen (10mg/ml PBS, 30min, 37°C) and analysed for antioxidative activity using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) test. The scavenging capacity was measured spectrophotometically by monitoring the decrease in absorbance at 517 nm.
Results: There were striking differences between various pollen species in their capacity of reducing the stable DPPH radical: *Corylus avellana* pollen extract had the highest potential followed by, *Alnus incana*, *Betula alba*, and *Artemisia vulgaris*. *Pinus sylvestris* and *Phleum pratense* exhibited nearly no antioxidative properties. Comparing only pollen from *Pinus sylvestris* (low allergenic) and pollen of *Phleum pratense* (high allergenic) it is interesting to note, that there is no correlation between antioxidative activity and sensitising potential from these two species. In situ exposure to various pollutants did not affect the antioxidative activity.
Conclusion: Pollen extracts from different species show disparate antioxidative capacity, which does not reflect their sensitising potential and which was not altered by SO2 and fine particles. Pollen grains release aqueous soluble substances with antioxidative activity, probably flavonoids. In summary our results contribute to the concept that pollen grains - apart from their natural mission - are far more than only allergen carriers.

P012

Twelve-Week Topical Treatment of Androgenetic Alopecia with a 5% Minoxidil Solution Promotes Hair Growth by Increasing the Ratio of Terminal/Vellus Hair and the Number of Ki67+ Proliferating Hair Follicle Cells in Horizontal Skin Sections

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Minoxidil has been shown to promote hair growth. The short-term effects of a 5% solution of minoxidil on the ratio of terminal/vellus hair and the proliferation marker Ki67 have not been examined before. We tested the effects of a 5% solution of minoxidil applied twice daily for 12 weeks on the scalp of 10 women and 9 men with androgenetic alopecia. Hair regrowth was evaluated by histologic analysis of paired 5 mm punch biopsy specimens for horizontal sectioning taken from the parietal region of the scalp from the right side on week 0 and the contra lateral side on week 12. In the histologic sections, the diameter of the hair shafts was determined in blinded fashion employing a micrometer grid; a hair shaft with a diameter of < 30 micrometer was defined as vellus hair, a diameter of > 30 micrometers as a terminal hair; data are presented as the ratio of terminal/vellus hair. Cellular proliferation in hair follicles was ascertained by Ki67 staining using the alkaline phosphatase method by counting the stained nuclei of 5 hair follicles in blinded fashion (Dako, Denmark). In addition, visual analysis was performed by scalp photography. We found the following ratios of terminal/vellus hair in women: 2.99 ± 0.49 (week 0, mean ± SE) vs. 4.74 ± 0.54 (week 12, $p < 0.05$, Wilcoxon test); in men: 2.89 ± 0.50 vs. 3.59 ± 0.54 ($p < 0.05$). Ki67 staining in hair follicles in women was: 8.04 ± 1.45 (week 0) vs. 9.59 ± 1.62 (week 12, $p < 0.05$); in men: 10.98 ± 1.53 vs. 11.18 ± 1.18 ($p > 0.05$, resp.); the combined values of men and women for Ki67 at week 0 were 9.35 ± 1.08 vs. 11.33 ± 1.10 at week 12 ($p = 0.02$). Scalp photography corresponded to increased hair growth after 12 weeks. These findings show that a twice daily, 12-week treatment with 5% topical solution of minoxidil enhances hair growth by increasing the ratio of terminal/vellus hairs in both men and women and by increasing Ki67 in hair follicles.

P013

Betulinic Acid Triggers Disruption Of Mitochondrial Membrane Potential (MMP) In Ejaculated Human Spermatozoa

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Objective: Disruption of the mitochondrial transmembrane potential is an integral part of type II apoptosis which has been demonstrated in human spermatozoa. Betulinic acid (BA) specifically induces apoptosis mediated by mitochondria. It is experimentally used for cancer treatment in humans. The aim of our study was to evaluate whether betulinic acid is able to induce apoptosis in ejaculated spermatozoa and if there is potential harm to fertility when used to treat other malignancies.
Design: A prospective study
Material and Methods: Semen samples from 10 healthy donors were allowed to liquefy for 15 min and washed after glass wool filtration. Thereafter the cells were incubated with BA to a final concentration of 60µg/ml for one hour which has been proven to reduce motility in previous studies. The proportion of cells with intact MMP was measured using a metachromatic lipophilic cationic dye. Intact MMP is displayed by a red fluorescence and disrupted MMP gives green signals. The staining results were analyzed by bi-color flow cytometry and the motility by computer aided sperm analysis (CASA, Cell Motion Analysis, Mika Medical Switzerland).
Results: Incubation with BA resulted in a significant decrease of sperm having intact MMP 37.6 ± 22.7 % vs. 93.8 ± 5.8 % ($p < 0.05$). Progessive motility (WHO a+b) was reduced after BA incubation immediately from 62.6 % to 41.4% and resulted in a further decrease after one hour to 27.5 % ($p < 0.05$). In comparison, the negative control sample showed an one hour motility of 58.4 % ($p > 0.05$).
Conclusion: It has been proved that BA induces type II apoptosis in human spermatozoa. Since BA is considered for induction of programmed cell death in human malignancies such as melanoma a possible interaction with germ cells has to be taken into account.

P014

Active Apoptosis Cascade In Impaired Human Spermatogenesis

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Objective: Apoptosis is thought to be one of the main regulators during spermatogenesis. Caspases (CP) are key enzymes of apoptosis when activated. The aim of our study was to evaluate CP8 and CP3 in line with BID, BAD and PARP in testes of infertility patients.Design: An immunohistochemistry study.Material and Methods: Activated CP8 and CP3, BID, BAD and cleaved PARP were investigated by immunohistochemistry (IH) on paraffin embedded testicular tissue derived from 10 infertility patients with different quality of spermatogenesis (Johnson Scores: 1,8 - 8,0). The number of positive spermatogonia, spermatocytes, Leydig cells and Sertoli cells per section was estimated as well as the intensity of staining (weak - intermediate - strong) and the intracellular distribution pattern. Results: All investigated antigens were found in a predominantly cytoplasmic staining pattern in spermatogonia, spermatocytes, Leydig- and Sertoli cells. Spermatides and mature testicular spermatozoa were free of staining. Active CP3 was detected in > 75% of all investigated cells, active CP8 in app. 50 % of germinal cells, most of the Sertoli Cells and hardly in Leydig cells. BID was present particulary in later stages of spermatogenesis and in > 75 % of Sertoli and Leydig cells, BAD was detected to similar amounts (50-75%) in all cell types. PARP cleavage was demonstrated most notably in early maturation stages and in Sertoli cells, but not in Leydig cells.Conclusion: We demonstrated that human testicular tissue houses activated CP8 and CP3. The presence of the initiator CP8 indicates that receptor mediated pathways may play a role for development of those cells. This corresponds with our earlier studies showing CD95/FAS and pro-CP8 on developing germ cells in conjunction with pro-CP3. Moreover, the existence of apoptosis regulators and cleavage of PARP in > 50 % of the germinal cells underlines the important role of apoptosis in spermatogenesis as a physiological but also pathophysiological process.Supported by a DFG grant (Gl199/1-4).

P015

The relevance of Chlamydial heat shock protein 60 in the diagnosis of Chlamydia trachomatis infections

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Objectives: Heat shock proteins (HSP) were first identified in cells after exposure to elevated temperature. HSP are highly conserved cellular stress proteins; their amino composition has not changed very much during evolution.The heat shock superfamily includes various families which are classified according to their molecular weight. The HSP are rapidly and distinctly synthesized by the cell in response to various physical, chemical and physiological stress-stimuli, in order to take on increased functions with regard to cell protection. Microbial HSP are dominant antigens and in humans extremly immunogenic. During an infection the microbes strongly increase their HSP synthesis, in order to protect themselves from the host's immunological defense mechanisms. In this connection HSP 60 becomes one of the most predominant bacterial proteins.
Material and methods: The presence of cHSP 60 to chlamydia was investigated in serum of 67 andrological patients, using a specific enzyme-linked immunosorbent assay (medac, GE Diagnostika, Germany). In addition the IgG and IgA antibodies against Chlamydia trachomatis have been determined in all patients (f=13; m=54). In this ELISA assay is used a synthetic peptid antigen of the immundominant region of the Major Outer Membrane Proteins (MOMP) from Chlamydia trachomatis.
Results: Specific antibodies against MOMP as well as against cHSP 60 could be found. There was a significant correlation between IgG and IgA antibodies against Chlamydia trachomatis and cHSP 60. On the basis of the results the patients were classified into 5 groups : I= early Chlamydia trachomatis infection; II= persisting latent Chlamydia trachomatis infection; III= persisting chronical Chlamydia trachomatis infection; IV= no evidence of Chlamydia trachomatis infection; V= cHSP 60 antibodies in the case of non-detectable Chlamydia trachomatis infection.
Conclusions: The recombinant ELISA for the determination of IgG antibodies against the chlamydial Heat Shock Protein 60 is a useful tool in the diagnosis of Chlamydia trachomatis infections, especially in the case of non-detected and insufficient treated infections.

P016

An automated method for the quantification and fractal analysis of immunostaining

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There is a growing trend towards the objective quantification of immunohistochemical staining. However, fully automated discrimination of immunostained particles is usually poor, and semi-automated or even interactive measurements have to be performed. In Tissue Counter Analysis (TCA), a recently developed and validated procedure, digital images of complex histologic sections are dissected into elements of equal size and shape, and digital information comprising grey level, colour and texture features is calculated for each element. In this study we assessed the feasibility of TCA for the quantitative description of amount and also of distribution of immunostained material in samples of normal skin. Two different primary antibodies were applied: Mouse Anti-Human Cytokeratin against CK (clone MNF116, 1:25, DAKO) and Mouse Anti-Human CD45 against LCA (clone 2B11+PD7/26, 1:50, DAKO). In a first step, our system was trained for differentiating between background and tissue on the one hand and between immunopositive and so-called other tissue on the other. In a second step, immunostained slides were automatically screened and the procedure was tested for the quantitative description of amount of CK and LCA immunopositive structures. Additionally, fractal analysis was applied to all cases describing the architectural distribution of immunostained material. Classification tree analysis of background versus tissue elements led to a correct classification of 98.0%. When the immunopositive components of the tissue elements were considered, CART analyses yielded to a correct classification of 97.0% of CK immunopositive elements and 88.9% of LCA immunopositive elements versus "other" tissue elements. T-test of the number and percentage of immunopositive elements revealed a significant difference between both antibodies ($p<0.001$). Evaluating the fractal geometry of each antibody, CK stained slides yielded a fractal dimension of 1.2+-0.1, whereas LCA stained slides produced a dimension of 0.4+-0.2 ($p<0.001$). In conclusion, our study shows that TCA is a fully automated and reproducible approach for the quantitative description of amount and also of distribution of immunopositive structures in immunohistology. In the future, TCA combined with fractal analysis will be used for the quantitative description of pathologic conditions.

P017

In situ zymography under neutral and acidic conditions demonstrates predominant acidic protease activity in bullous pemphigoid

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The inflammatory process in bullous pemphigoid finally resulting in subepidermal blistering is not completely understood. Different proteases have been implicated to play a role, i.e. different members of the matrix metalloprotease family and several serine proteases, which are both normally most active at neutral pH and are thought to play a key role in extracellular matrix degradation. However, recently it became evident, that acidic lysosomal proteases participate in extracellular matrix degradation outside the cell under certain circumstances.
We investigated the proteolytic activity in bullous pemphigoid biopsies by in-situ gelatin zymography under neutral and acidic conditions. In situ zymography visualizes the respective net proteolytic activity in the tissue and allows matching the location of activity to the histological picture. In normal skin no gelatinolytic activity is seen at both conditions. At neutral pH only 8/15 biopsies exhibited a mainly moderate gelatinolysis, but under acidic conditions all specimens (15/15) showed a prominent or strong gelatinolytic activity. Moreover, proteolytic activity was generally stronger at acidic than at neutral conditions. Inhibition experiments using class specific inhibitors indeed revealed, that cysteine and aspartate proteases participate in the observed gelatinolytic activity. In summary, our results suggest an important role for acidic proteases in proteolytic process resulting in blister formation.

P018

Parakeratotic keratinization is associated with a reduced epidermal expression of N-methyl-D-aspartate receptors (NMDAR1)

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Ionotrope glutamate receptors of the N-methyl-D-aspartate (NMDA) receptor type are expressed on keratinocytes and influence the intracellular calcium concentration. The importance of NMDA-receptors in pathological processes in the skin is unknown. Therefore, epidermal distribution patterns of NMDA-receptors are examined in skin diseases with parakeratotic cornification (Psoriasis vulgaris, Verrucae vulgares, actinic keratoses).
The expression of NMDA-receptors (R1-components) in paraffin-embedded normal epidermis (n=22), Psoriasis vulgaris (n=21), Verrucae vulgares (n=23) and actinic keratosis (n=16) was examined by digital image analysis. To compare the patient groups with one another, a ratio (NMDA-Ratio) was formed from the values of the Stratum granulosum and the Stratum basale (SG/SB).
NMDA-receptors could be observed in the Stratum basale and Stratum spinosum, but a significantly higher receptor density occurred within the Stratum granulosum in healthy skin. This distribution pattern was basically also present in the other dermatoses examined. Thereby, the occurrence of parakeratosis in Psoriasis vulgaris and in actinic keratoses, but not in Verrucae vulgares, was characterized by a significant reduction of the NMDA-ratio (reduced expression of NMDAR1 in the upper epidermis).
The expression of NMDA-receptors depends especially on formation of the Stratum granulosum. In diseases in which no Stratum granulosum is formed, the lack of NMDA-receptors could be of functional importance and lead to parakeratotic cornification.

P019

High Sensitivity And Reproducibility Of Immunohistochemistry By Micro-Agitation

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Objective: We have examined the practicability, reproducibility and analytical sensitivity of classical immunohistochemistry (IHC) versus IHC by micro-agitation.
Patients and Methods: Tow different monoclonal antibodies Ki-67 (proliferation marker) or p53 (tumor suppressor marker) were used to compare classical IHC versus micro-agitation IHC. Consecutive paraffin sections of patients with non-melanoma skin cancer were used in this study. Reproducibility was examined using specimens of four different patients in three independent experiments with antibodies against Ki-67 and p53. Analytical sensitivity was analyzed by serial dilutions in two independent experiments with both methods.
Results: IHC by micro-agitation was realizable without destroying the tissue. The new technique was consistent and reproducible and no background staining was observed. The primary antibodies Ki-67 and p53 could be used at higher dilutions (5-10 times) by micro-agitation compared with classical IHC.
Conclusion: Micro-agitation can be used for immunohistochemistry, it was reproducible, highly sensitive, and antibodies could be used in higher dilutions. Further analyses with other antibodies using this technique are warranted.

P020

Clinically equivocal melanocytic skin lesions with features of regression-a dermoscopic-pathologic study

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OBJECTIVES:To evaluate diagnostic significance and histopathologic correlates of blue-white structures(BWS)seen by dermoscopy in a series of clinically equivocal melanocytic skin lesions that were excised.DESIGN:Each lesion was photographed clinically and dermoscopically.All lesions were reviewed for the degree, type and location of BWS, Dermoscopically and the presence of melanosis and/or fibrosis, histopathologically. RESULTS:All included lesions (N=158) showed partial or focal regression histopathologically.135 (85.4%)lesions were diagnosed as melanocytic nevi(complete histopathologic interobserver agreement), whereas 23(14.6%)were defined as equivocal because at least one out of four pathologists was diagnosing the given lesion as melanoma.Only one lesion was diagnosed as melanoma by all four pathologists.The majority of nevi exhibited blue areas(84.4%)with a central distribution(57%)and involving less than 50% of the lesion's surface(89.6%).By contrast, 78.3% of equivocal lesions revealed a combination of white and blue areas with an irregular distribution(60.9%)and involving more than 50% of the lesion's surface(47.8%).CONCLUSION:Using degree and type of BWS an algorithm was constructed that can be applied for the management of lesions exhibiting dermoscopic features of regression.

P021

Induction of differentiation in a human skin organ culture model by telomere-homologue oligonucleotides (T-oligos) in comparison to 1,25 dihydroxy-Vitamin D3 (VitD3) and all-trans retinoic acid (ATRA).

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Treatment with various agents can modulate differentiation in human skin. Here we examined whether treatment with small single-stranded DNA-fragments, homologue to the 3'overhang of human telomeres, termed telomere-homologue oligonucleotides (T-oligos) which have been shown to induce a differentiated phenotype in certain cell types as well as to modulate various DNA-damage responses, can induce markers of differentiation in human skin organ culture. Treatment was compared to known modulators of differentiation, such as 1,25 dihydroxy-Vitamin D3 (VitD3) and the retinoid all-trans retinoic acid (ATRA).
Punch biopsies (6mm) of normal human skin were taken from volunteers and immediately incubated (with the epidermis at air-liquid interface) with either 20µM T-oligo, 1µM ATRA or 10nM VitD3. After 48 hours the treated samples were fixed and stained for cytokeratin 1 and 10 (CK1, CK10), the cornified envelope proteins involucrin and filaggrin and the proliferation marker Ki-67.
Compared to PBS (diluent) treated samples, treatment with the T-oligo lead to an enhanced staining for CK 1 and CK 10 in the suprabasal epidermal layers. VitD3 also lead to a stronger staining for these cytokines than diluent, however less than T-oligo, while treatment with ATRA resulted in a marked decrease in CK1 and 10 expression. Involucrin and filaggrin were more prominent in the granular and horny layers only after the treatment with VitD3, while T-oligo and ATRA treatment resulted in a reduced staining intensity. No effect was seen in the Ki-67 expression for either treatment.
Here we demonstrate in a 'supravital' skin model that T-oligos can influence differentiation in human skin similar to known regulators of differentiation, such as VitD3 and ATRA. In accordance with data from in vitro experiments in cell cultures, where cells undergo cell-cycle arrest and adapt a more differentiated phenotype (or undergo apoptosis), markers of a differentiated skin phenotype are also induced in this model.
Further studies are required to elucidate the spectrum of actions and cellular interactions for T-oligos and investigate their potential therapeutic use in skin diseases that show alteration in cellular differentiation.

P022

Altered apoptosis and cell cycling of mast cells in bone marrow lesions of patients with systemic mastocytosis

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Mastocytosis is a rare disorder (OMIM 154800) characterized by accumulation of mast cells in tissues, particularly in skin and bone marrow. To investigate whether an altered regulation of mast cell proliferation or apoptosis might be involved in the pathogenesis of mastocytosis, expression of the apoptosis-preventing molecules bcl-2 and bcl-xL, the proliferation marker Ki67, the cell cycle-controlling molecules p53 and p21, and distribution of apoptotic ISEL-positive cells were studied by immunohistochemistry in bone marrow infiltrates of 14 patients with systemic mastocytosis and compared to 2 control patients with reactive bone marrow characterized by an unspecific hyperplasia of mast cells. Of the 14 patients with systemic mastocytosis, 12 patients had indolent systemic mastocytosis (ISM) and 2 patients suffered from systemic mastocytosis with an associated clonal hematologic non-mast cell lineage disease (SM-AHNMD). In patients with mastocytosis, expression of bcl-xL protein was significantly enhanced in mast cell infiltrates of the bone marrow, compared to control patients. Comparing patients with ISM and SM-AHNMD, only patients with ISM were found to express high levels of bcl-xL, whereas the 2 patients with SM-AHNMD failed to overexpress bcl-xL. In addition, expression of p21 was slightly increased in all patients with mastocytosis, but not in controls. In contrast, reactivity for bcl-2, Ki67, and p53, and ISEL staining were weak or absent in mastocytosis patients as well as controls. Since we have previously shown that bcl-2, but not bcl-xL, is overexpressed in cutaneous mastocytosis lesions, these results suggest that survival of bone marrow mast cells in this disease may be differentially regulated compared to skin mast cells. Our data further support the concept that alterations in the control of apoptosis and cell cycling may contribute to accumulation of mast cells in mastocytosis.

P023

Identifying hereditary *mut S homolog 2* defects in dermatological patients: Novel genotype-phenotype correlation with impact on internal cancer prevention

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The neoplasm of the sebaceous gland is the skin manifestation of MTS (Muir-Torre syndrome), a variant of the internal cancer predisposition syndrome HNPCC (hereditary nonpolyposis colorectal cancer). We performed mutation analysis in a sample of 41 unrelated patients with MTS or a sebaceous neoplasm. 37 patients had been pre-selected for DNA mismatch repair deficiency in tumour tissue by proof of high microsatellite instability or loss of mut S homolog 2/mut L homolog 1 protein expression. In 27 (66%) of the 41 patients, we detected germline mutations in the DNA mismatch repair genes. In contrast to pure HNPCC patients, significantly more mutations in the *mut S homolog 2* gene were detected among these patients: 25 (93%) mutations were located in *mut S homolog 2*, only two in *mut L homolog 1* Our findings should have consequences for mutation detection protocols in MTS patients or HNPCC patients who have family members with MTS. Interestingly, six (22%) of the mutation carriers do not meet the strict clinical Bethesda criteria for diagnosing HNPCC, nor do their families. Applying only these current clinical criteria would have resulted in these patients being overlooked. In view of internal cancer prevention, the MTS phenotype should therefore be regarded as a highly specific indicator for hereditary DNA mismatch repair deficiency, especially caused by *mut S homolog 2* defects.

P024

Changes of Gene Expression in PMA Stimulated Murine PAM212 Cells Using Micro Array Analysis

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Protein kinase C-activating phorbol-12-myristate-13-acetate (PMA) is known to induce proliferation and differentiation in keratinocytes. The impacts of PMA on keratinocyte gene regulation, on signal transduction and on immunity have not yet been established. We investigated changes in gene profiling after PMA stimulation by treating a spontaneously transformed PAM212 keratinocyte cell line with PMA and analysing gene expression with a customised murine micro array technique. 24 hours after PMA treatment, we extracted total RNA through reverse transcription and generated cDNA probes. Fragmented cDNA was hybridised to the micro array containing oligonucleotide probe sets representing more than 400 transcripts. The hybridised gene chips were scanned and genes were filtered which produced signals stronger than an arbitrary cut-off point for florescent signal intensity (more than 1.4 fold increase or decrease). PMA stimulation induced strong up-regulation in scavenger receptor class B member 2 involved in signal transduction, beta chain of interleukin 2 receptor regulating autoimmunity, lymphotoxin B important for anti-parasitical immunity, caspase 4regulating apoptosis-related cysteine protease, regulatory subunit 2 of cyclin-dependent kinase 5 (p39), and envoplakin a scaffold protein in the epidermal cornified envelope, which is important for differentiation. Additionally, the expression of genes of the innate immunity system was affected by PMA, ß-defensin-1 and -5 were down-regulated, whereas ß-defensin-3 and -4 and lipopolysaccharide binding protein showed up-regulation. Our results indicate that PMA treatment modulates a subset of genes important for epidermal differentiation, apoptosis, and immunity.

P025

Differential expression of transglutaminases in LEKTI deficient skin may explain the impaired epidermal barrier in Netherton syndrome

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Netherton syndrome is an autosomal recessive congenital ichthyosis featuring chronic inflammation of the skin, hair anomalies, atopic manifestations and epidermal hyperplasia with an impaired epidermal barrier function such as a markedly increased transepidermal water loss. The disease is caused by mutations in the SPINK5 gene encoding the serine proteinase inhibitor LEKTI. The reason why LEKTI deficiency is associated with an impaired epidermal barrier in Netherton syndrome is unknown. Sequence analyses of SPINK5 allowed us to identify two known and three novel mutations predicting a substantial truncation of the LEKTI polypeptide in 7 Netherton syndrome patients from 5 different families. We developed a monoclonal antibody giving a strong signal for LEKTI in the stratum granulosum of normal skin and demonstrate absence of the protein in Netherton epidermis. As transglutaminases are key enzymes for the assembly of the conified envelope and thus are directly involved in establishing the epidermal barrier, we looked into transglutaminases in LEKTI deficient skin of Netherton syndrome. Transglutaminase-1 activity was present throughout almost the entire suprabasal epidermis in NTS, while in normal skin it is restricted to the stratum granulosum. In contrast, immunostaining for transglutaminase3 was absent or faint. Interestingly, comparable to the altered pattern in psoriatic skin the epidermis in NTS strongly expressed the serine proteinase inhibitor SKALP/elafin and the antimicrobial protein human ?-defensin 2. These studies demonstrate LEKTI deficiency in the epidermis at the protein level and a differential expression of transglutaminase-1 and -3, which may account for the impaired epidermal barrier in Netherton syndrome.

P026

Variations in the genes encoding the peroxisome proliferator-activated receptors a and ? in psoriasis

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The three peroxisome proliferator-activated receptor (PPAR) subtypes a, ß (or d), and ? belong to the group of nuclear receptors that act as ligand-activated transcription factors. Recently, expression of PPARa and ? in keratinocytes has been demonstrated, and ligands of PPARa and ? were found to enhance epidermal maturation and protect against cutaneous inflammation. There is first evidence for a possible role of PPARs in psoriasis, as expression of PPARa and ? is decreased in lesional skin and treatment with PPAR? agonists improves psoriatic keratinocyte pathology in vitro and in vivo. We performed a case-control study to search for possible associations between variations in the genes encoding PPARa and ? and psoriasis. Seven variations in these genes were analyzed in 192 patients with chronic plaque-type psoriasis and 330 healthy controls by PCR-based methods. No association between any of the investigated PPAR variants and psoriasis was found. Our findings argue against a significant contribution of the investigated PPAR variations to the genetic basis of psoriasis.

P027

Comparison of SAGE™ expression profiles of inflammatory skin diseases

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As part of a large scale approach to identify drug target candidate genes and diagnostic/prognostic marker genes linked to inflammatory skin diseases and skin cancers, we have generated the complete expression profiles of normal skin, psoriasis, atopic and contact dermatitis by SAGE™ technology. In addition, a SAGE™ library for Lichen ruber is being generated.
The SAGE™ libraries (30.000 tags each) were constructed using RNA of pooled patient samples. The comparison of the SAGE™ libraries for diseased versus healthy skin revealed a number of differentially expressed genes. At a p-value smaller 0.01 we found several genes differentially expressed exclusively in psoriatic skin, atopic dermatitis or contact dermatitis. In addition, a number of differentially expressed genes was identified not only induced or repressed in one of the diseases but shared between either two or all three of the diseases.
To differentiate between genes expressed only in individuals or subgroups, and putative universal marker genes for inflammatory diseases, we used the PIQOR™ array technology to hybridize single patient samples versus a common reference on PIQOR™ Skin1.0 arrays. Otherwise, putative marker genes allowing a new pathological classification might be missed or individual differences in gene expression misinterpreted as universal.
The comparison showed a good overall concordance of the SAGE™ and the PIQOR™ results, while some differences were found that underline the importance of comparing pooled versus single patient samples.

P028

Genome Scans Provide Evidence for Keloid Susceptibility Loci on Chromosomes 2q and 7p

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Keloids are proliferative fibrous growths that result from an excessive tissue response to skin trauma. They often occur sporadically, but in some families a genetic predisposition to keloids has been observed. Here we studied two families with an autosomal dominant inheritance pattern of keloids. One African-American family showed a high degree of variability in the extent of keloid formation between family members, whereas the second family from Japan showed a pattern of full penetrance and formation of only small keloids. We performed a genome wide linkage search for genes predisposing to keloid formation in these two families. We identified linkage to chromosome 2q (maximal two-point LOD-score 3.01) for the Japanese family. The African-American family showed evidence for a keloid susceptibility locus on chromosome 7p (maximal two-point LOD score of 3.16). The observed locus heterogeneity in autosomal dominant keloid disease is consistent with the clinical heterogeneity of this scarring disorder. Dense microsatellite analysis in these two loci was performed and candidate genes were identified. This study provides the first genetic evidence for keloid susceptibility loci and serves as a basis for the identification of responsible genes.

P029

The contribution of corneodesmosin to androgenetic alopecia

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Androgenetic alopecia (AGA, male pattern baldness) is the most common form of hair loss in men and women. Significant AGA affects well over one half of the adult male population. It is characterized by a loss of hair from the scalp that follows a defined pattern. The development of AGA is androgen-dependent and of genetic origin. To date, a very limited number of studies have been performed concerning the molecular genetic basis of AGA. Recently, we identified corneodesmosin (CDSN) as the responsible gene for hypotrichosis simplex of the scalp (HSS). HSS is an autosomal dominant form of isolated alopecia causing almost complete loss of scalp hair, with onset in childhood. Interestingly, among the known single gene hair disorders, the phenotype in HSS resembles the phenotype in androgenetic alopecia. Furthermore, the histological picture of scalp biopsies from patients with HSS has many features of androgenetic alopecia such as miniaturized follicles of the vellus type and absence of scarring. Following the identification of the gene for HSS, we thoroughly examined CDSN for a possible contribution to the development of androgenetic alopecia in a sample of 168 trios, 177 single probands and 111 controls. We selected 21 intragenic variants, including 12 SNPs causing amino acid exchanges. Preliminary results at the level of individual polymorphisms in CDSN did not show any significant association with AGA. Further statistical analyses are currently underway.

P030

Functions of NF-?B essential modulator in epidermal keratinocytes

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NF-?B essential modulator (NEMO) or I kappa B kinase ? is an X-linked gene mapped to chromosome Xq28. Mutations of NEMO cause incontinentia pigmenti of the type 2 (IP2) which is an X-linked dominant disorder characterised by skin inflammation and disturbance of skin pigmentation and is associated with a variety of malformations of hair, nails, teeth, heart, eyes and the central nervous system. Abnormalities in the development of sweat glands, hair and teeth is also a feature of hypohidrotic or anhidrotic ectodermal dysplasia which has been shown to be associated with hypomorphic mutations of NEMO.
We have performed histological analysis of the skin from mutant mice with conditional ablation of NEMO selectively in the epidermis. These mice develop a severe skin phenotype during the first days of their lives. We have analysed this phenotype and have found dramatic alterations in proliferation, differentiation and apoptosis of epidermal keratinocytes as well as severe skin inflammation and disturbed morphogenesis. Comparative analysis of mice with epidermis specific deletion of NEMO or I kappa B kinase 2 indicate that these components of the I kappa B kinase complex have both overlapping and distinct functions in the regulation of epidermal morphogenesis and skin homeostasis.

P031

Interferon regulatory factor 2 is associated with type 1 psoriasis

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Type 1 interferon is known to trigger flares of psoriasis. Recently, hypersensitivity to type 1 interferon signaling was found to cause a psoriasis-like skin disease in mice deficient for the transcription factor interferon regulatory factor 2 (IRF2). The human IRF2 gene is located at a previously identified candidate psoriasis susceptibility locus on chromosome 4q (PSORS3 at D4S1535). Therefore, we tested association of psoriasis with IRF2. We generated a sample consisting of 157 families with a total of 521 individuals. Five novel microsatellite markers were developed and typed, and complemented with three known markers to yield a set of eight markers spaced within 600 kb around the IRF2 gene, three of which are located in the gene. We detected association of IRF2 with type 1 psoriasis at two markers in the IRF2 gene. Haplotype sharing analysis confirmed statistically significant association of IRF2 with type 1 psoriasis ($p = 0.0017$; $p_{corr} = 0.03$). The 921G/A SNP in exon 9 was found to obliterate a predicted exon splice enhancer in an allele-specific manner. There was a suggestive increase of homozygosity for the splicing-deficient allele in type 1 psoriasis patients. Our data identify IRF2 as a potential susceptibility gene for psoriasis.

P032

Single cell comparative genomic hybridization (SCOMP) in dermatological research

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Molecular cytogenetic techniques usually require large amounts of DNA. Unfortunately, the genomic DNA that can be obtained from highly interesting clinical samples, such as small melanocytic lesions, is very limited. We therefore tested a recently introduced amplification method, termed SCOMP, for genomic DNA to study chromosomal aberrations in malignant melanoma. So far we applied the amplification protocol to the DNA of 36 single melanoma cells that were isolated from the peripheral blood or sentinel lymph nodes and to 32 formalin-fixed paraffin-embedded tissue samples of primary tumors and distant metastases after laser microdissection. As controls we used seven single lymphocytes and three microdissected stroma samples as well as DNA from cell line cells with known genomic aberrations. After amplification all samples were used for comparative genomic hybridisation (CGH) to evaluate chromosomal aberrations on a genome wide scale. We found that successful CGH depends on several variables such the age of tissue sample, immunocytochemistry and the applied fixation protocol resulting in success rates ranging from 30 to 90%. The presented data show that SCOMP is a robust and valuable tool to study samples comprising single to few cells but that the influence of clinical sample preparation has to be carefully evaluated.

P033

Treatment of Human Skin with Retinoic Acid Highly Induces Aldehyde Dehydrogenase 6 (ALDH6)

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Retinoids are widely used for topical and systemic treatment of skin disease. The active form of retinoids is retinoic acid, which serves as ligand for nuclear retinoic acid receptor and activates transcription. In order to study the effect of retinoic acid, we treated human keratinocytes in a skin equivalent model with 10-6 M all-trans-retinoic acid (ATRA) and compared the expression profile with an untreated control sample by DNA chip hybridization. One of the highest ATRA induced genes found was the aldehyde dehydrogenase 6 (ALDH6) also named retinaldehyde dehydrogenase 3 (RALDH3) or aldehyde dehydrogenase 1A3 (ALDH1A3). When human keratinocytes were treated with ATRA a rapid induction of this gene could be seen with a maximum of 30-fold up-regulation as tested by real time PCR. This strong induction was verified by Northern blot and in situ hybridization of skin equivalents. Interestingly other members of this family of enzymes such as ALDH1A1, ALDH1B1, ALDH2 and ALDH3A2 were not induced by retinoic acid. The effect of retinoids in activating ALDH6 is very likely restricted to epithelial cells as retinoid-treatment of fibroblasts under the same conditions resulted in no up-regulation of its expression. As ALDH6 is highly induced by retinoic acid we hypothesize that this enzyme plays a role in counterbalancing the effects of retinoic acid by metabolising it to retinaldehyde.

P034

The molecular basis of porphyria cutanea tarda in Chile: Identification and functional characterization of mutations in the uroporphyrinogen decarboxylase gene

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The porphyrias are heterogeneous disorders arising from predominantly inherited catalytic deficiencies of specific enzymes in heme biosynthesis. Porphyria cutanea tarda (PCT) results from a decreased activity of uroporphyrinogen decarboxylase (URO-D), the fifth enzyme in heme biosynthesis. The disorder represents the only porphyria that is not exclusively inherited as a monogenetic trait. In PCT, at least two different types can be distinguished: Acquired/sporadic (type I) PCT, in which the enzymatic deficiency is limited to the liver and inherited/familial (type II) PCT, which is inherited in an autosomal dominant fashion with decreased URO-D activity in all tissues. Here, we characterized the molecular basis of PCT in Chile and identified eight mutations in eighteen previously unclassified PCT families by PCR, heteroduplex analysis, and automated sequencing. To study the causality of these mutations in causing disease, in vitro expression of all novel missense mutations was performed. Our results indicate that the frequency of familial PCT in Chile is approximately 50%, thus, to our knowledge, representing the highest incidence of familial PCT reported to date. The data further emphasize the molecular heterogeneity in type II PCT and demonstrate the advantages of molecular genetic techniques as a diagnostic tool and for the detection of clinically asymptomatic mutation carriers.

P035

Role of junctional adhesion molecules (JAMs) in leukocyte recruitment into skin

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A hallmark of inflammation is leukocyte extravasation, which is mediated by adhesion molecules expressed on leukocytes and endothelial cells in a stepwise process. In contrast to the first steps (rolling, activation, adhesion), molecular mechanisms of leukocyte transendothelial migration are less understood. Recently, much attention has been attributed to JAMs in leukocyte transmigration. JAMs are adhesion molecules of the Ig gene superfamily. JAM-1 is located at intercellular junctions of endothelial cells and is ligand for the ß2-integrin LFA-1. JAM-2 and -3 are also expressed by endothelial cells and bind VLA-4 or Mac-1, respectively. Since JAM-1 antibodies were shown to inhibit leukocyte migration in a model of experimental meningitis, we sought to assess if JAM-1 and -2 contribute to recruitment of leukocytes in cutaneous inflammation. For this purpose mice were sensitized and challenged with DNFB. After development of delayed-type hypersensitivity (DTH, 24h) ears were snap frozen. Immunofluorescent quantitation of JAM-1 and -2 expression in histological sections showed a constitutive expression in almost all vessels. No further increase could be observed in DNFB- vs. vehicle-treated ears. However, this does not rule out an involvement of JAM-1 in cutaneous inflammation, since JAM-1 shows a phosphorylation-depended binding and translocation from intercellular junctions to the apical side of the endothelium following an inflammatory stimulus. Immunoprecipitation from mouse-ear lysates with JAM-1Ab did not reveal any change in overall serine phosphorylation. A blockade of JAM-2 alone 2 hours before DNFB-challenge (4 mg/KG bw anti-JAM-2 i.p.) had no effect on DTH as determined by increased ear thickness (isotype control Ab: 28.0±8.0 vs. 32.3±25.3 mm x 10^{-3}; n=6 for both groups). In conclusion, JAM-1 and -2 are constitutively expressed in murine vasculature and their expression pattern seems unchanged upon inflammation. Blocking of JAM-2 does not influence a DTH reaction in mice. A blockade of JAM-1 alone, or a combined blockade of JAM-1 and -2 may be required to influence leukocyte extravasation.

P036

UV-induced regulatory T cells inhibit the effector phase of contact hypersensitivity only upon injection into the area of challenge

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Epicutaneous application of haptens onto UV-exposed skin induces hapten-specific T suppressor cells, now renamed regulatory T cells (Tr). Intravenous (i.v.) injection of Tr inhibits the induction of contact hypersensitivity (CHS) when injected into naive recipients, but not the elicitation when injected i.v. into sensitized recipients. In contrast, when UV-induced Tr were injected intracutaneously (i.c.) into the ears of sensitized mice, elicitation of CHS was suppressed. To further characterize the nature of UV-induced Tr, depletion studies using magnetobead separation was performed. Depletion of either CD4+ or CD25+ fractions resulted in the loss of transfer of suppression upon i.v. or i.c. injection, indicating that UV-induced Tr belong to the subgroup of CD4+CD25+ Tr. Inhibition of challenge upon i.c. injection of Tr was hapten-specific since injection of dinitrofluorobencene (DNFB)-specific Tr into the ears of oxazolone (OXA)-sensitized mice did not affect the challenge with OXA. However, when ears of OXA-sensitized mice were injected with DNFB-specific Tr and painted with DNFB before OXA challenge, CHS response was suppressed. This indicates that the activation of Tr is hapten-specific, however, once they are activated their suppressive activity is non-specific (bystander suppression). The inhibitory effect correlated with the local expression of interleukin-10 as demonstrated by PCR. Accordingly, suppression could be blocked by i.c. injection of an antibody which neutralizes interleukin-10. Depletion studies also revealed that UV-induced Tr express the lymph node homing receptor L-selectin (CD62L). Expression of CD62L was functionally relevant since incubation with MEL14, an Ab which blocks CD62L, prevented transfer of suppression when injected i.v. into naive mice. In contrast, FACS analysis demonstrated that Tr do not express the ligands for the skin homing receptors E- and P-selectin. Thus, we conclude that UV-induced Tr though being able to inhibit T effector cells do not suppress the elicitation of CHS upon i.v. injection since they do not migrate into the skin due to the expression of lymph node homing but not of skin homing receptors.

P037

Role of Dendritic cells in establishment and reprogramming of tissue selective CD8+ T cell trafficking to gut versus inflamed skin.

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Effector/memory T cells show tissue selective trafficking by expression of distinct adhesion molecules like E-selectin ligand (ESL) and chemokine receptors like CCR4/10 for skin homing and integrin a4ß7 and CCR9 for homing to small intestine. We and others have shown that these homing subsets are rapidly generated during first antigen contact and that tissue specific DC promote the corresponding tissue homing phenotype. However, the crucial factors for the generation and the stability of established homing characteristics are not known, yet. Using TCR transgenic P14 CD8+ T cells primed in vitro by DC derived from skin versus gut and transferred into wildtype mice, we have adressed the following questions:
1) Can in vitro priming with DC from different tissues instruct CD8+ T cells for homing to inflamed skin in vivo ?
2) How stable is the differential upregulation of the homing markers ESL and a4ß7 in vitro after in vivo transfer over time ?
3) After induction of specific homing phenotype: Is reprogramming possible? Do we find switching between or integration of different, tissue selective trafficking patterns ?
Our preliminary data indicate that tissue selective homing characteristics are stable for at least 3 weeks. Restimulation of skin homing CD8+ P14 T cells (ESL high) with mesenteric lymph node DC in vitro leads to upregulation of a4ß7 integrin, indicating flexibility of established tissue homing patterns. In turn, Langerhans cells promote high expression of the skin homing marker E-selectin ligand on gut homing (a4ß7 high) T cells. Interestingly, only a subset (appr. 50%) of the T cells changed their expression pattern of these homing markers in vitro. These preliminary results suggest a high flexibility only in a subset of effector T cells.

P038

Defect of $CD8^+CD28^-$ cytotoxic T regulatory cells in patients with atopic dermatitis

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In addition to $CD4^+$ $CD25^+$ T cells, $CD8^{hi+}$ $CD28^-$ cytotoxic T cells exert important regulatory functions. They eliminate mature antigen presenting cells (APC) and B cells in an antigen specific manner using their perforin (Perf)-granule system. In addition, maturation of APCs is prevented by these cells. We asked, if $CD8^{hi+}$ $CD28^-$CTLs may have a defect in patients with exacerbated atopic dermatitis (AD).Ficoll-isolated peripheral blood mononuclear cells (PBMC) from 28 AD-patients and 16 healthy controls (HC) were fixed, permeabilized, triple-stained against Perf, CD8, CD28 and analyzed in a FACScan flow cytometer. In addition, in 20 individuals, PBMC were depleted of monocytes and activated by (i) ionomycin and PMA, (ii) anti-CD3 antibody (MEM92) (iii) or kept under control conditions for 0, 30, 60, 90, 120, 180 min. At time point zero, significantly fewer $CD8^{hi+}$ CTLs expressed CD28 in AD (26 ± 12%) as compared to HC (49 ± 11%; $p < 0.0001$). In addition, $CD8^{hi+}$ $CD28^-$ AD-CTLs expressed significant fewer Perf (27 ± 18% versus HC: 60 ± 26%; $p < 0.0005$). Setting this value at 100%, a Perf-hyperreleasability was detected: After 30 min, 37 ± 14% (PMA/ionomycin), 41 ± 7% (CD3-crosslinking), 92 ± 16% (unstimulated) of $CD8^{hi+}$ $CD28^-$ still stained Pef^+ in AD as compared to 51 ± 8%, 58 ± 19%, 95 ± 9%; $p < 0,05$, $< 0,05$, $> 0,05$, respectively, in the HC-group.Whereas no functional defect in $CD4^+$ $CD25^+$ regulatory T cells is reported in AD, this defect in regulatory CTLs, namely numerical reduction, Perf-depletion and -hyperreleasability, may help to understand immuno-dysregulation in AD.

P039

Use of HLA transgenic mice and predictive algorithms for the identification of a new HLA-DR3 restricted epitope from the TRP2 (Tyrosinase related protein 2) melanoma differentiation antigen

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Antigen-specific cytotoxic CD8+ T lymphocytes (CTL) are effective mediators of destructive anti-tumor immunity. In vivo induction of a potent cytotoxic immune response requires the interaction of at least three cellular players: dendritic cells, antigen-specific CD4+ T helper (Th) lymphocytes and antigen-specific CTL. The Th cells not only contribute to primary CTL sensitisation and proliferation via DC activation and cytokine secretion but also to the induction of CTL memory. Therefore active immunotherapy (e.g. peptide vaccination) of melanoma should target antigen-specific CTL and Th cells. In contrast to HLA-class I antigenic peptides only a few HLA-class II restricted epitopes from melanoma antigens are known. We used computer algorithms and HLA-DR3 transgenic mice to identify epitopes from the TRP2 melanoma antigen. Three potential HLA-DR3 restricted epitopes were predicted from the TRP2 protein sequence. Of the corresponding synthetic peptides one sequence (Pep1) exhibited strong and another sequence (Pep2) intermediate binding affinity. The third peptide was a low affinity binder. Peptide immunization of HLA-DR3 transgenic mice repeatedly activated Pep2-specific T cells. Processing of the Pep2 sequence from the TRP2 antigen was demonstrated by immunization of HLA-DR3 transgenic mice with recombinant protein. This led to the identification of Pep 2 as a natural TRP2 epitope. Analysis of Pep3 immunogenicity in human cell culture systems demonstrated that T cells specific for Pep2 could also be activated by in vitro peptide stimulation of human PBL from normal HLA-DR3+ individuals. At present, recognition of HLA-DR3+ tumor cells by Pep2-specific T cells and Pep2-specific immune responses in melanoma patients are analysed.

P040

Papillomavirus-Like Particle Amyloid Beta Vaccine Induces Strong Antibody Response

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The non-infectious virus-like particles (VLP) composed of major capsid protein L1 of papillomaviruses are successfully being tested in humans as vaccine to prevent anogenital HPV infection and associated neoplasia, in particular cervical cancer. In addition, chimeric VLP have been generated that incorporate foreign or self-antigens, and immunizations have induced high levels of (auto-)antibodies, whereas immunizations with linear peptides were less effective. Antibodies were long-lasting and functionally active in experimental animal models.
Using the baculovirus system we have generated recombinant bovine papilloma virus type 1 (BPV1) L1 protein, with 9 amino-acid (aa) N-terminal peptide of amyloid beta (Abeta) inserted into an immunogenic VLP surface loop (Abeta-VLP). Electron micrographs (negative stain) demonstrated spherical particles of approximately 50 nm in diameter indicating that the chimeric protein self-assembled into capsomeric VLP. Expression of the inserted Abeta peptide was verified by Western blotting using monoclonal antibody to N-terminus of Abeta. This peptide shows aa sequence identity in human and rabbit representing a self-antigen for both species. To examine whether Abeta-VLP are capable to break tolerance, NZW rabbits were immunized with four injections of Abeta-VLP, 50 microgramm each, or wild-type VLP as control using Freunds adjuvant. After five months no apparent sign of toxicity was observed. By ELISA the rabbit inoculated with Abeta-VLP developed a strong antibody response (titer of 10.000) to Abeta peptide 1-9, whereas the control rabbit did not. Reactivity was specific for the Abeta epitope and blocked by homologous, but not control peptides of similar length. These data support papillomavirus-like particles as a novel tool to overcome tolerance to central self-antigens with the potential of inducing therapeutically useful autoantibody responses in humans. This hypothesis is currently evaluated by in vitro and in vivo models of Alzheimer´s disease.

P041

Subsets of murine blood monocytes in infection

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In some infections blood phagocytes transport microbes and enable their entry into certain organs. The exact identity of these leukocytes is not known. While in the human system the existence of different monocyte subsets is acknowledged, characterization of murine monocyte subsets is just emerging. Our goal was to identify monocyte subsets in the mouse and to analyse their response to infections with *Leishmania major (L.major)* and *Listeria monocytogenes (List.monocyt)*.
By extensive FACS analyses and cell sorting we found that mouse monocytes can be distinguished into three subsets by their differential expression of Ly-6C, i.e. Ly-6C$^{hi\ /\ med\ /\ low}$, and, similarly, of CD43, CD11c and CD62L. Each of these subsets is phagocytic, expresses M-CSF R (CD115), and develops into macrophages or DC depending on the growth factors. By eliminating blood monocytes with clodronate-loaded liposomes and monitoring their repopulation as well as their kinetics in bone marrow we found that monocytes reappearing in the circulation are exclusively of the Ly-6C^{hi} subset and that this is the subset released from bone marrow. In blood Ly-6C is then down regulated during maturation. Chronic infection with *L. major* and acute infection with virlulent, but not avirulent (*? hly*) *List.monocyt* produced a significant increase in immature Ly-6C^{hi} monocytes, resemblingthe inflammatory "left shift" of granulocytes. By FACS analysis we showed that Ly-6C$^{med/hi}$ monocytes are the ones preferentially recruited to inflamed tissues. This subset also harbored the majority of bacteria and, unexpectedly, sometimes also *L. major*. Importantly, in systemic Listeriosis we found recruitment of highly infected Ly-6C^{hi} monocytes into the brain. In leishmaniasis the parasitic load of Ly-6C^{hi} cells was low and they were not recruited to the brain. The fate of Ly-6C^{low} monocytes is not known yet.Thus: i) there are distinct subsets of murine monocytes with Ly-6C$^{med/hi}$ cells being preferentially recruited in inflammation; ii) these subsets can harbor different pathogens; iii) the Ly-6C^{hi} subset transports *List.monocyt* into the brain; iv) infection with different microbes produces different signals for release and recruitment of monocytes.

P042

Early cytoskeletal rearrangement during Dendritic Cell maturation enhances synapse formation and Ca^{2+} signalling in CD8+ T-cells

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The interplay between dendritic cells (DC) and T-cells is a dynamic process critically depending on DC maturation. Ca^{2+}-influx is one of the initial events occurring during DC / T-cell contacts. To determine, how DC-maturation influences DC / T-cell contacts time-lapse video microscopy was established using TCR transgenic CD8+ T-cells from P14 mice. DC maturation shifted DC/T-cell contacts from short-lived interactions with transient Ca^{2+}-influx in T-cells to long-lasting interactions and sustained Ca^{2+}-influx of 30 min. and more. Follow-up of DC / T-cell interactions after 2h using confocal microscopy revealed that long-lasting Ca^{2+}-responses in T-cells were preferentially associated with the formation of an immunological synapse involving CD54 and H2-kb at the DC / T-cell interface. Such synapse formation preceeded MHC or B7 upregulation, since DC developed into potent Ca^{2+}-stimulators 7h after initiation of maturation. Instead, the enhanced capacity of 7 h matured DC to induce sustained Ca^{2+}-responses in CD8 T-cells is critically dependent on the polarization and rearrangement of the cytoskeleton, as shown by *Clostridium difficile* toxin B inhibitor experiments. These data indicate that already very early after receiving a maturational stimulus DC display an enhanced cytoskeletal activity resulting in the rapid formation of immunological synapses and effective CD8+ T-cell stimulation.

P043

Identification of a melanoma-associated chondroitin sulfate proteoglycan (MCSP) peptide recognized by CD4+ T lymphocytes on human melanoma cells.

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The identification of tumor antigens recognized by cytolytic CD8+ T cells (CTLs) on human tumor cells has opened new avenues in cancer immunotherapy. There is consensus, that the induction of both, tumor-specific CTLs and CD4+ T helper cells is necessary for an optimal antitumor immunity. So far, only a few tumor-specific helper T cell epitopes have been described so far. We therefore have focused our research on the identification of melanoma antigens recognized by CD4+ T cells. One interesting candidate antigen is the human melanoma-associated chondroitin sulfate proteoglycan (MCSP), which is expressed on > 90% of human melanoma tissues and induces strong humoral responses in mice. In the present study, we describe the induction of MCSP-specific CD4+ T cell clones from the peripheral blood of a healthy human donor and the subsequent identification of the T cell epitope which is located in the core protein. The identified peptide was presented to the T helper cells by HLA-DR11 molecules, which are expressed by approximately 13% of Caucasians. The T cells directly recognized HLA-matched MCSP-expressing melanoma cells and produced high amounts of IFN-gamma, a cytokine with important antitumoral effects. To the best of our knowledge, this is the first MCSP-derived T cell epitope described and it should be useful for melanoma immunotherapy.

P044

***In vivo* effect of CD4+CD25+ regulatory T cells on hapten induced leukocyte-endothelium interaction in the murine model of skinfold chamber**

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In vitro studies have indicated that the suppressive capacity of CD4+CD25+ T cells requires cell-cell contact, assumedly independent on cytokines. The mechanism of *in vivo* suppression remains largely unknown. In order to elucidate the mechanism of the *in vivo* suppressive function of regulatory T cells in allergic contact dermatitis, we analyzed the microcirculatory changes directly at the site of inflammation using intravital fluorescence microscopy. Therefore, C57/BL6 mice were implanted with skinfold chambers on their entire back. This permits the microscopic quantification of the leukocyte influx in the arterioles, the capillaries and the post-capillary venules after applying the hapten *in vivo*. 6 days after sensitization with TNCB, FITC labeled CD4+CD25- or CD4+CD25+ T cells were i.v. injected just before challenging with TNCB (or carrier substance) directly on the skinfold chamber. Using in vivo leukocyte fluorescence marker Rhodamin, it was possible to investigate both the endogenous leukocytes and the injected T cells during the inflammatory reaction. Under baseline conditions, the majority of circulating leukocytes did not interact with the endothelium, challenging with hapten caused a significant increase of slow-rolling and adhesive leukocytes within four hours correlated with an increasing edema. Injection of syngeneic CD4+CD25- T cells did not influence the reaction of the allergic contact dermatitis whereas CD4+CD25+ regulatory T cells eliminated the leukocyte-endothelium interaction completely from the beginning. These results indicate that CD4+CD25+ regulatory T cells prevent influx of effector T cells into inflamed tissue and point to a novel mechanism that regulatory T cells use to modify immune reactions.

P045

Characterization of mutational mechanisms causing lack of HLA class I molecule expression on different melanoma cell lines

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Downregulation or even total loss of HLA-class I molecule expression enables tumor cells to escape HLA-restricted CTL effector activity. The molecular nature of these escape mechanisms has to be characterized in detail in order to develop immunotherapies which circumvent tumor defense strategies. Therefore we analyzed the mechanisms contributing to the loss of HLA-class I presentation on four different melanoma cell lines: Mel 249, Mel 499, Mel 505 and Mel 592. None of the cell lines exhibited surface presentation of HLA-class I molecules after interferon-g treatment as verified by FACS analysis. Re-expression of HLA-class I molecules after transient transfection with a beta2-microglubolin expression plasmid was clearly detectable for the two cell lines, Mel 505 and Mel 592, but not for Mel 249 and Mel 499 which might be due to a low transfection rate. This led to the assumption that a mutation in the beta2-microglubolin gene might be casual for the HLA-class I negative tumor cell phenotype. In order to define the molecular mechanisms we first analysed beta2-microglubolin expression at the RNA level. Total RNA from all cell lines and the positive control cell line Hela was isolated followed by RT-PCR analysis. According to the control, a beta2-microglubolin specific cDNA fragment was detectable for Mel 249 and Mel 499 cells. Sequence analysis of the PCR products revealed a 2 base pair deletion in exon II of the beta2-microglubolin gene for Mel 249 whereas an insertion of intron sequences of different length between exon I and exon II was observed for Mel 499. In contrast no beta2-microglubolin specific cDNA product could be obtained for Mel 505 and Mel 592. Therefore we tried to amplify genomic DNA sequences of Mel 505 and Mel 592 choosing different primer pairs for synthesis of overlapping PCR fragments covering the exons and the exon/intron transitions of the beta2-microglubolin gene. In contrast to the control we could not amplify genomic DNA sequences from Mel 505 and Mel 592, suggesting an extensive deletion of genomic sequences. Further PCR and Southern Blot analysis as well as fluorescence

P046

Identification of Toll-like receptors and characterisation of the signal transduction pathway during *Candida albicans* infections

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Recognition of pathogens by the host is an essential first step in the induction of an immune response. TLRs are specialized for recognition and binding of pathogen associated molecular patterns (PAMPs) that are unique for pathogens.
Here we analyzed the functional relevance of TLR2 and TLR4 for recognition of the pathogenic fungus *Candida albicans*. We used HEK 293 cells transfected with human TLR2 or TLR4-expression plasmids, respectively, and TLR2, TLR4, TLR2/4-deficient murine macrophages in order to present evidence that TLR2 and TLR4 are involved in recognition of *C. albicans*. Stimulation of wild-type mouse macrophages with wild-type *C. albicans* resulted in activation of NF-?B in a dose-dependent manner. Whereas TLR2 expression was essential for NF-?B activation after stimulation with antimyotics-treated and untreated *C. albicans*, TLR4 expression was found to be dispensable for the recognition of *C. albicans* if the macrophages were pretreated with IFN-?. In the *in vitro* system utilizing HEK293 cells, TLR2 was found to be essential for *C. albicans* recognition, whereas TLR4 was of less relevance. Significant NF-?B dependent reporter gene expression was observed only after stimulation with antimycotics-treated *C. albicans* and not with untreated *C. albicans*. The differences in the reaction pattern between untreated *C. albicans* and *C. albicans* treated with cytoplasmic membrane-interacting antimycotic points to differences in PAMP recognition due to changes of the membrane structure.
Analysis of the signal transduction cascades following *C. albicans* recognition demonstrated phosphorylation and degradation of I?B-a. Moreover we can show, that Jun N-terminal kinases (JNK) and c-Jun, a component of the transcription factor AP-1 that binds and activates transcription at TRE/AP-1 elements, as well as the mitogen activated protein (MAP) kinases p38 and p44/42 are activated. It remains to be elucidated in future studies, if there might be a crosstalk talk between NF-?B and AP-1.

P047

Langerhans Cells, Dermal Dendritic Cells And Monocyte-Derived Dendritic Cells Are Distinct Subsets of Myeloid Dendritic Cells Defined By Diverse Functional Properties

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Dendritic cells (DC) are known to be the most potent antigen presenting cells with the unique ability to induce naive T cell responses in vivo. Clinical investigators are already using DC in human immunotherapy trials with the aim to fight disease by stimulating the own immune system. Discussion persists, however, with respect to the type of DC which is best for this purpose. We therefore compared the functional properties of three types of myeloid DC, monocyte-derived dendritic cells (moDC), CD34+derived Langerhans cells (LC), and CD34+ derived dermal-interstitial dendritic cells (DDC). We find that LC and DDC are superior to moDC in the induction of a T cell response both in non-specific allostimulatory assays and most importantly in specific autologous cytolytic assays in which CTLs against tumor or viral antigens were induced up to 2-5-fold higher levels than with moDC. Furthermore, we find that despite their better capacity to induce a T cell response, LC and DDC phagocytose 3-4-fold less than moDC which scavenge huge amounts of apoptotic material. Surprisingly, although the inducible peptide of IL-12, IL-12p40, is produced by all three subsets after the application of a maturation stimulus, the active IL-12p70 dimer is produced only by moDC and never detected with LC and DDC.
In this work we show that LC and DDC are superior stimulators of T cell responses compared to moDC. Further our data suggest differences in the mechanism by which TH1 responses are mediated by DDC and LC versus moDC. Both aspects might be interesting with respect to the clinical application of DC within immunotherapy trials.

P048

The antimicrobial peptide Dermcidin is not induced in epidermal keratinocytes under inflammatory skin conditions

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Antimicrobial peptides (AMPs) are important effector molecules of innate immunity protecting epithelial surfaces of multicellular organisms. In human skin two classes of antimicrobial peptides - the b-defensins and the cathelicidins - are produced by keratinocytes primarily under inflammatory conditions. In contrast, Dermcidin (DCD), a recently discovered AMP with broad spectrum activity, is expressed in eccrine sweat glands and transported via sweat to the epidermal surface. In the present study we investigated whether DCD expression is induced under inflammatory conditions in epidermal keratinocytes. By immunohistochemistry using an anti-DCD antiserum lesional skin of the inflammatory dermatoses atopic dermatitis, psoriasis and lichen planus was analysed. Whereas DCD is constitutively expressed in eccrine sweat glands of all skin biopsies, we found that independent of the type of the inflammatory skin lesion DCD protein expression is not induced in human epidermal keratinocytes. In contrast ß-defensin 2 (HBD-2) is expressed in epidermal keratinocytes of inflammatory human skin, but not in keratinocytes of healthy human skin. Moreover, we examined whether DCD RNA expression is induced in cultured human keratinocytes, melanocytes and skin tumor cells. Upon stimulation of the cells with TPA, TNF-a, LPS or H_2O_2, DCD-mRNA expression was not detected in primary skin cells, but in tumor cells. These results indicate that unlike human cathelicidins and ß-defensins which are inducible peptides that primarily function in response to injury and inflammation, DCD is exclusively part of the constitutive innate defense of human skin. By modulating surface colonization, DCD may help to prevent local and systemic invasion of pathogens.

P049

Role of CD137 ligand in differentiation and activation of human immature dendritic cells

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The initiation and maintenance of an immune response requires activation and maturation of dendritic cells, allowing for subsequent interaction with antigen specific T cells. DC differentiation is initiated by the engagement of surface molecules such as FcRs and CD40 that also mediate additional T cell activation. Like CD40 ligand, CD137 is known for its T cell costimulatory capacity, mediated via signaling through its receptor on the T cell surface. Here, we investigate the expression and function of the corresponding CD137 ligand in human monocyte-derived dendritic cells (MoDCs) and its interaction with T cells.
Immature MoDCs express the CD137 ligand already at baseline level. Its activation led to accelerated differentiation, with up-regulation of the maturation markers CD83, CXCR4, and CCR7, as well as of HLA-DR and the T cell costimulatory molecule CD86. In addition, an associated secretion of TNF-a, and IL-12 was observed.
MoDCs differentiated by crosslinking of the CD137 ligand asses an enhanced T cell costimulatory capacity. They induced T cell proliferation and primed T lymphocytes to release Th1 associated cytokines in an autologous antigen-specific MLC.
CD137 ligand signaling provides therefore a new physiological means of human MoDC
activation and T cell costimulation, with strong evidence for important immunoregulatory function at the interface of adaptive immunity

P050

Soluble Cd83 Inhibits Experimental Autoimmune Encephalomyelitis (EAE) In Mice

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CD83 is an Ig superfamily member that is up-regulated on the surface of dendritic cells (DCs) during maturation and has been widely used as a marker for mature DC. Recently, we reported the recombinant expression of the extra-cellular IG domain of human CD83 (hCD83ext). Using this soluble hCD83ext molecule it was shown for the first time that CD83 has also a functional role. Indeed, using this soluble CD83, allogeneic- as well as specific CTL proliferation could be blocked in a concentration dependent manner in vitro. Here we report the functional analysis of soluble CD83 in vivo, using murine experimental autoimmune encephalomyelitis (EAE) as a model. Strikingly, only three injections of soluble CD83 prevented the paralysis associated with EAE almost completely, while untreated control mice developed strong paralysis. This therapeutic effect was dose-dependent and the best inhibition was observed at a concentration of 100 µg / injection. Furthermore, even when the EAE was induced for a second time, CD83 treated mice were protected, indicating a long lasting suppressive effect. In summary, this study strongly supports an immunosuppressive role of soluble CD83, thereby indicating its therapeutic potential in the regulation of immune disorders in vivo.

P051

Two distinct subpopulations of $CD25^+$ regulatory T cells in a model of infectious tolerance

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Control of self-reactive T lymphocytes by regulatory T cells (Tregs) is essential for induction and maintenance of peripheral tolerance. It has been shown that human Tregs consist of several heterogenous populations which use different mechanisms to suppress T cell responses including cell contact-dependent as well as contact-independent mechanisms. We have characterized two subsets of $CD25^+$ regulatory T cells isolated from peripheral blood of healthy human volunteers. Both subsets express GITR (Glucocorticoid induced TNF-receptor family related protein) but can be distinguished by the expression of either the integrin a4ß1 or a4ß7. Upon activation, both populations express FoxP3, recently described as a key factor of murine Tregs. Activated $a4ß1^+$, as well as $a4ß7^+$ $CD25^+$ Tregs, suppress the activation of conventional $CD4^+$ T cells in a cell contact-dependent manner. Furthermore, both subsets convert the anergized $CD4^+$ T cells into secondary Th suppressor cells (Th_{sup}) through a mechanism of infectious tolerance. This process is accompanied by Treg-induced expression of FoxP3 in Th_{sup}. The induced Th_{sup} inhibit T cell activation in a cell contact-independent manner through soluble factors. However, the Th_{sup} induced by $a4ß1^+$ or $a4ß7^+$ T regulatoy cells showed different patterns in the production of inhibitory cytokines. Th_{sup} induced by $a4ß1^+$ $CD25^+$ Tregs produce mainly TGF-ß like Th3 cells, whereas $a4ß7^+$ $CD25^+$ Tregs induce Tr1-like Th_{sup} which secrete high amounts of IL-10. Our findings indicate that downregulation of autoreactive T cell responses through naturally occurring $CD25^+$ Tregs is a two-step mechanism. First, $CD25^+$ Tregs suppress T cells locally through a cell contact-dependent mechanism followed by the conversion of the suppressed $CD4^+$ T cells into secondary Th suppressor cells. These induced Th_{sup} then possibly inhibit immune reactions systemically via production of soluble factors such as IL-10 and TGF-ß.

P052

Desmoglein 3 (Dsg3)-Specific T Regulatory 1 (Tr1) Cells In Patients With Pemphigus Vulgaris (PV) And Healthy Individuals

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There is increasing evidence that autoreactive T helper (Th) cells are involved in the regulation of auto-antibody (auto-Ab) in PV an autoimmune bullous skin disorder primarily associated with auto-Ab against Dsg3. In light of the recent detection of Dsg3-autoreactive T cells in PV patients and healthy donors (which do not produce auto-Ab!), the goal of this study was to identify and characterize Dsg3-responsive T regulatory cells in both populations. Utilizing MACS cytokine secretion assay, Dsg3-responsive IL-10-secreting Tr1 cells were isolated both from PV patients and healthy carriers of the PV-associated HLA class II alleles, DRB1*0402 and DQB1*0503. The Dsg3-specific Tr1 cells were characterized by the secretion of IL-10, TGF-ß and IL-5 upon Ag stimulation, their inhibition of the proliferative response of Dsg3- and TT-responsive Th clones in an Ag-specific (Dsg3) and cell number-dependent manner and their proliferative response to IL-2 but not to Dsg3 or mitogenic stimuli. Moreover, their inhibitory effect was blocked by Ab against IL-10, TGF-ß and paraformaldehyde fixation. These observations strongly suggest 1) Dsg3-responsive Tr1 cells are present in PV patients and healthy individuals, 2) their growth requires the presence of IL-2, and 3) they exert their Dsg3-dependent inhibitory function by the secretion of IL-10 and TGF-ß. In conclusion, the identified Dsg3-responsive Tr1 cells may be involved in the maintenance of peripheral tolerance against Dsg3.

P053

Human CD4+CD25+ T cells regulate Th2 cytokine production depending on the allergen concentration.

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Recently, we have shown that regulatory CD4+CD25+ T cells (Treg) are present and functional in most atopic patients with allergic rhinitis and are able to inhibit Th1 as well as Th2 cytokine production of CD4+CD25- T cells while no suppression apart from the Th1 cytokine IFN-gamma was observed in 20 % of the investigated patients. This study was set out to further analyze under which conditions Treg are able to regulate Th2 cytokine production and to extend the observations to wasp venom allergic patients. For this purpose, CD4+CD25- T cells from grass pollen or wasp venom allergic donors were stimulated alone or in the presence of Treg with autologous mature monocyte-derived dendritic cells which were pulsed with a concentration of the respective allergen resulting in an optimal proliferation index of CD4+ T cells (1-5 µg/ml) and a ten fold lower concentration. Using wasp venom at an optimal concentration during DC pulse, Treg from about 80% of the examined wasp venom allergic patients show almost the same proliferative responses and cytokine production (IL-4, IL-5, IL-10, IFN-gamma) compared to CD4+CD25- T cells even after initiation of venom immunotherapy when the immune responses was generally downregulated. However, using ten fold lower concentrations of wasp venom for DC pulse, proliferation and cytokine production of Treg was low and they suppressed Th1 and Th2 cytokine production by CD4+CD25- T cells. These data demonstrate that in allergic diseases the function of Treg is dependent on the concentration of the respective allergen with different thresholds for individual patients and allergens.

P054

Optimizing the Antigen loading of Dendritic Cells with Exogenous Peptides

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The central role of dendritic cells (DC) in the immune system and their unique potency to induce tumor-specific killer and helper T cells has been demonstrated in numerous studies and is today unequivocal. Therefore, DC-based immunotherapy represents one of the most promising approaches to fight cancer and since the first vaccination study in 1996 numerous trials have been performed with more than 30 DC-based vaccination trials published only in the past 3 years. In principle, antigen can be delivered to DC by various strategies, but most commonly HLA class I or II restricted peptides derived from defined tumor antigens have been used. Because peptides can be readily obtained in clinical grade quality, are easily standardized and facilitate the immuno-monitoring during clinical trials, they can still be considered as gold standard of DC antigen loading.
Nevertheless, several issues concerning the use of peptide-loaded DC still have to be addressed. In the present study we carefully analyzed different parameters such as peptide concentration, stability of HLA/peptide complexes on immature (i-DC) versus mature-DC (m-DC) or antigen competition in order to optimize the loading of DC with HLA class I and II peptides.

P055

Immunological consequences of the sentinel lymph node biopsy

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In the last years the sentinel lymph node (SLN) excision was established as a standard staging procedure for solid tumors such as breast cancer or melanoma. Besides the diagnostic value, the excision of the SLN may also be therapeutic beneficial. However, the removal of this secondary lymphoid tissue responsible for the initial induction of specific immune responses - the SLN is the place where antigen presenting cells initially meet naive T cells - also raises concerns; the excision of the SLN could be detrimental for the immune reaction against the tumor. Indeed, tumor-specific T cells are detectable in the SLNs.
In the present work we performed a thorough immunological workup of a patient developing two consecutive melanomas. An excision biopsy of a pigmented lesion obtained in June 2000 from the left shoulder of the patient turned out to be a superficial spreading melanoma. Histological analyses of the removed SLN revealed a lymph node micro-metastasis. In 2002 upon preventive examination an ulcerating superficial spreading melanoma in association with a congenital nevus was detected. The subsequently removed SLN did not display any metastatic spread. The immunological analyses of SLN2000, primary tumor (pT)2002 and SLN2002 displayed, that albeit the SLN2000 actually harbored T cells recognizing the tumor, the very same T cell clones present in the removed SLN are nevertheless maintained in other compartments since they were providing the inflammatory infiltrate of the second melanoma. Moreover, using real time PCR a specific T cell clone was analysed longitudinal and could be detected among peripheral blood lymphocytes and in a subsequent removed metastases. Thus, the presented case is illustrating the substantial role of the SLN for cellular immune responses to melanoma, but also that the excision of the SLN does not seem to prevent the recruitment of T cells originally harbored in this lymphoid organ for immune responses to subsequently evolving melanomas.

P056

The protease inhibitor Hurpin is overexpressed in inflammatory and neoplastic skin diseases

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Hurpin is a serine protease inhibitor which was isolated from the keratinocyte cell line HaCaT. Expression of Hurpin was shown to be restricted to the skin and to be transiently downregulated by UV light. Overexpression of Hurpin in keratinocytes confers resistance to UV-induced apoptosis. Recently, we showed that Hurpin is a potent inhibitor of the lysosomal cysteine proteinase cathepsin L. Initial studies by RT-PCR showed that Hurpin is overexpressed in lesional psoriatic skin. To further study the expression of Hurpin in healthy skin and different skin diseases, we generated monoclonal antibodies against Hurpin. Two antibodies were employed to optimize the immunohistochemical detection of Hurpin in paraffin and cryosections. In comparison to healthy skin, we found an upregulated expression of Hurpin in sections from psoriatic skin, atopic eczema, lupus erythematosus, actinic keratoses, basal cell carcinoma and squamous cell carcinoma and melanoma. The expression was further quantified by PCR using RNA from biopsies. Normalized to beta-actin, we found Hurpin to be upregulated to various extents in individual diseases.
Differential gene expression of Hurpin in skin diseases and healthy skin might be important for processes including immunity, inflammation and apoptosis.

P057

Th-1 cytokines in alopecia areata pathogenesis: IFN-? is essentially involved while IL-2 plays a minor role

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Alopecia areata (AA) was regarded as a Th-1 mediated autoimmune disease of the hair follicle, because the Th-1 cytokines IFN-? and IL-2 are expressed in lesional AA-skin. Recently we have shown, that also the Th-2 cytokine IL-10 is expressed in skin infiltrating leucocytes in AA of C3H/HeJ mice and that IL-10-/- mice are resistant to the development of AA, pointing towards an involvement of Th-2 cytokines in AA pathogenesis. To prove the functional relevance of Th-1 cytokines in AA, we induced AA in IFN-? -/- mice and in IL-2+/- mice by grafting lesional AA skin from AA-affected mice.
After experimental induction by skin-grafting 90% of normal C3H/HeJ mice (control) developed AA, while none of the IFN-? -/- mice and 47% of IL-2+/- mice developed AA.
Immunhistochemistry showed dense peri- and intrafollicular infiltrates of CD4+ and CD8+ cells and an aberrant expression of MHC-I on hair follicle epithelium in control mice with AA, while infiltrates and MHC-I-expression were absent in IFN-? -/- and IL-2+/- mice without AA.
FACS-analysis of skin-draining lymph node cells revealed no significant differences in the number of CD4+ and CD8+ lymphocytes, but a significant reduction of T-cell activation markers and co-stimulatory molecules in IFN-? -/- mice (CD25, CD69, CD28, CD86) and IL-2+/- mice (CD25, CD28, CD80) and a reduced expression of pro-inflammatory cytokines in IFN-? -/- mice (IL-6, TNF-a) and IL-2+/- mice (IL-4, IL-5, IL-6, IFN-ß, TNF-a).
Our data show that IFN-?g-/- mice are resistant to the development of AA, pointing towards an essential role of IFN-?g in the pathogenesis of AA. Flow cytometry and immunohistochemistry demonstrate that IFN-? is irreplaceable for activation of autoreactive T-cells in the lymph nodes. A pathogenetic role for IL-2 in AA could also be demonstrated, but the expressiveness of our data is limited because only heterozygous IL-2+/- mice were available for these experiments. FACS-analysis and immunohistochemistry point towards an involvement of IL-2 in the activation of autoreactive T-cells in the lymph nodes.

P058

Novel Mutation in ZAP70 revealed by Chromosomal Mapping and Sequencing of Candidate Genes of a Hyper IgE Mouse Mutant Generated by Genome Wide Mutagenesis.

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Laboratory animals carrying mutations are of great value to study the biological function of genes. Using a genome wide mutagenesis approach (ENU-Mouse-Mutagenesis), we have generated and identified a number of mouse mutants with altered immunglobulin levels. One of these mutants displays a hyper IgE phenotype and additional immunological abnormalities. Flowcytometric analysis of pB, LN and spleen lymphocyte subpopulations revealed dramatically reduced T cell numbers, while B cell counts were normal. Thymocyte analysis of mutants indicate a block in late T cell differentiation where numbers of CD4, CD8 double positive cells appeared to be normal, while single positive cells where almost absent. Functional challenge showed an absence of humoral immune response in homozygots while the response in heterozygote was normal. Chromosomal mapping revealed a link to a 2.1 cM region localized on chromosome 1. Sequencing of selected candidate genes revealed a novel point mutation at the kinase domain of zeta chain associated protein kinase 70 kD (ZAP70). ZAP70 is involved in early TCR signalling and in thymocyte development. In humans mutations in the ZAP70 kinase domain have been reported in patients with servere combined immuno-deficiency (SCID) characterized by the absence of peripheral CD8+ T cells and the presence of non-functional CD4 T cells. In conclusion, we have identified a novel mouse mutant with a combined phenotype of hyper IgE, a late block in T cell differentiation and an almost complete absence of peripheral T cells. Chromosomal mapping and sequencing allowed us to identifiy a novel ZAP70 mutation . Additional characterization of ZAP70 signalling may reveal novel genotype/phenotype relationships and may lead to a novel animal model for human SCID.

P059

Role of the CD137 ligand in human mast cells

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Mast cells are not only key players in allergic reactions but are also very potent effector cells in innate and adaptive immune responses. To fulfil these actions mast cells have to communicate with other cells of the immune system, especially with T cells.
Although it is well-known that mast cells and T cells interact with each other, little is known about the proteins that enable this communication. The CD137 receptor and its ligand represent candidates, which may mediate such a cell-cell interaction. The CD137 receptor is expressed on activated T cells and is known for its T cell costimulatory capacity.
In this study, we investigated the expression and function of the corresponding CD137 ligand protein in human mast cells. We found a constitutive mRNA and protein expression of the CD137 ligand in cells of the immature human mast cell line HMC-1 and in human skin mast cells. Activation of mast cell via the CD137 ligand mediated a selective cytokine response in both cell types, since a release of TNF-a was found whereas IL-8 secretion was not induced. In HMC-1 an increase of IL-6 and the upregulation of the adhesion molecule ICAM-1 were also observed. These findings suggest an intriguing and as yet unidentified role of the CD137 receptor / ligand costimulatory system as a mediator of T cell- mast cell interactions.

P060

Gene expression analysis of psoriasis vulgaris using custom made microarrays

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Psoriasis is a T-cell mediated inflammatory skin disease of unknown etiology. Several aspects including genetic predisposition, antimicrobial defense mechanisms, and autoimmunity may be involved in the development of this complex disorder. Recent technology provides cDNA- or oligonucleotide based targeting of more than 60,000 genes simultaneously. While intriguing in it's possible power to detect genes of yet unknown significance, the amount of data gathered by these chips constitutes a serious biostatistical challenge. As a result, sensitivity and power of experiments based on large-scale expression chips often become diluted due to biometrical necessities.
In our study we constructed microarrays with selected targets to be used in an interdisciplinary approach to inflammatory diseases trying to identify causative pathophysiological traits. The arrays contain 400 target genes particularly involved in inflammatory pathways. Of these, about 125 genes were found to be expressed in psoriatic plaques by means of mRNA extraction from 5 mm punch biopsies of lesional skin from 10 patients with chronic plaque type psoriasis. Biopsies of nonlesional skin from the same patients served as intraindividual controls. In addition skin biopsies from healthy individuals served as normal controls for cutaneous gene expression. Real-time RT-PCR was used for verification of results. Among those genes being prominently expressed in lesional skin were genes of the S-100 family as well as genes of the epidermal differentiation complex, several keratin genes, and matrix metalloproteinases. Regarding immunoregulatory and signaling genes, different MAP kinases, NF ?B, CD44, several interleukins, and TNF a related genes could be identified. Expression of HLA-C was upregulated, as were beta-defensin-2, chemokine- and toll-like receptors.
There was considerable variation of gene expression pattern between different patients, that could partially be correlated to the type and the acuity of the disease. In conclusion, our method provides accurate results for the investigation of different types and stages of psoriasis, and reveals new potential insights into the pathophysiology of the diesease.

P061

Human epidermal Langerhans cells differ from monocyte-derived Langerhans cells in CD80 expression and in secretion of IL-12 and IL-10 after CD40 crosslinking

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In spite of the fact that Langerhans cells (LCs) are often referred as the best studied members of the family of dendritic cells some questions still remain unsettled, including their phenotypical and functional maturation after appropiate stimulation. Blood-derived monocytes can be used to generate cells that mimic epidermal LCs from human skin (MoLCs). LC-characteristic Birbeck granules are induced when GM-CSF, IL-4 and most important TGF-ß1 are added to the cell culture. Here we report about analyses of MoLCs and LCs concerning their maturation-induced antigen expression andcytokine release. To achieve comparable cell populations, they both were isolated by CD1c cell sorting resulting in high purity of CD1a/DR positive cells. CD40 was found in freshly enriched cells at similar level. After stimulation with CD40 ligand, both LCs and MoLCs acquired CD83 and increased CD86. High CD80 expression was exclusively detected in CD1c + MoLCs. HLA-DR and ICAM-1 expression was found in all populations but at different intensities. Triggering CD40 upregulated the potency of LCs and MoLCs to stimulate CD4 T cells. But only activated MoLCs simultaneously released IL-12p70 and anti-inflammatory IL-10 after 18 h. More than TNF-a, TGF-ß1 upregulated the corelease of these cytokines and after application of the danger signal LPS, high amounts of IL-12p70 and IL-10 were detected. Our data indicate that both TGF-ß1-generated MoLCs as well as epidermal derived LCs display no maturation arrest concerning their CD83 and CD86 maturation. However, the failure of LCs to release significant IL-12/ IL-10 even after CD40 triggering and LPS stimulation, demonstrates that the induction of a Th1 response requires more than a T cell and danger signal danger for effective stimulation of epidermal LCs. This may reflect a resistance against activation thereby protecting the organism from undue inflammations.

P062

Activation of MAP kinase p38 is required for cell cycle-mediated regulatory function of T cells.

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Previous results demonstrated that human IL-10-treated dendritic cells (DC) induce anergic $CD4^+$ T cells with antigen-specific regulatory function. Analysis of the cell cycle in anergic T cells revealed high levels of the cdk inhibitor $p27^{Kip1}$ resulting in an arrest of cell cycle progression in the G1 phase as compared to activated control T cells. Here, we studied the interaction between cell cycle regulation and signal transduction of MAP kinases in anergic T cells as compared to optimally stimulated T cells (cocultured with mature DC). Immunoprecipitation assays demonstrated a reduced activity of the MAP kinases JNK1/2 and ERK1/2 in anergic T cells as compared to control T cells. In contrast, we observed a markedly enhanced and sustained activity of the MAP kinase p38 during primary culture and after restimulation. Additional analysis of the kinase MAPKAP2/3, the downstream substrate of p38, revealed a significantly increased activity, suggesting an altered critical pathway of signal transduction in anergic T cells. Blocking of MAP kinase p38 using the specific inhibitor SD203580 completely impaired the induction of anergy in T cells as demonstrated by proliferation assays, IL-2 production and cell cycle analysis. Furthermore, western blot assays showed a downregulation of the G1 phase specific cdk inhibitor $p27^{Kip1}$ after inhibition of p38 resulting in a cell cycle progression in T cells cocultured with IL-10-treated DC. More importantly, after addition of the p38-inhibitor the T cells also lost their antigen-specific regulatory function, indicating that modulation of the cell cycle is involved in the suppressor activity of regulatory T cells. Thus, our data show that activation of the MAP kinase p38 is required for the cdk inhibitor $p27^{Kip1}$-induced cell cycle arrest which was a critical role for the regulatory function of T cells induced by IL-10-treated DC.

P063

HLA class II transgenic mice as a model for the human CD4+ T cell response to desmoglein 3 in pemphigus vulgaris

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Pemphigus vulgaris (PV) is a life-threatening autoimmune disease primarily caused by IgG autoantibodies against the cadherin-like adhesion molecule desmoglein 3 (Dsg3). There is a strong association between the susceptibility to PV and the HLA class II alleles DRB1*0402 and DQB1*0503. Several groups have identified Dsg3-reactive Th1 and Th2 cells in patients with active PV. Analysing the immunodominant T cell epitopes of Dsg3 presented by the disease-associated HLA class II molecules is an important step in the investigation of the pathogenesis of this autoimmune disease. To address this problem experimentally we are using HLA class II transgenic mice carrying the PV-associated HLA haplotype (DR0402-DQ8) and the human CD4 receptor. In addition these mice are deficient for murine MHC class II (I-Aß-/-). The HLA-DR0402/DQ8-/hCD4-transgenic mice are immunized with purified recombinant protein containing the extracellular domain (EC1-5) of the desmoglein 3 molecule. T cell hybridomas are generated by fusion of activated Dsg3-reactive T cells from the draining lymph nodes with the TCRa/ß-/- variant of the BW5147 thymoma cell line. Growing T cell hybridomas are propagated in selection medium and tested for IL-2 production in response to stimulation with intact Dsg3 protein. In order to determine their peptide-specificity Dsg3-reactive T cell hybridomas are then stimulated with pools of 10-12 overlapping peptides spanning the mature protein. We obtained T cell hybridomas recognizing two peptides, which are located in the extracellular domain 4 and 5 of the Dsg3 protein, so far. T cell hybridomas are an ideal tool to further characterize the epitopes of these peptides using N- and C-terminal truncated versions as well as alanin substituted variants. The effect of an incomplete T cell receptor activation by altered peptide ligands can also be analysed. Using these HLA class II transgenic mice will provide a systematic approach to identifying immunodominant epitopes of the autoantigen Dsg3 and to designing new immunotherapeutic strategies for this autoimmune disease.

P064

B cells are not required for effector T cell activation during contact hypersensitivity and tolerance to contact allergens.

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Low zone tolerance (LZT) to contact allergens is induced by epicutaneous application of low, subimmunogenic doses of contact allergens resulting in an inhibition of contact hypersensitivity (CHS) which, in contrast, occurs after sensitization with immunogenic doses of haptens. To evaluate the role of B cells as antigen-presenting cells for the activation of effector T cells of CHS (Tc1) and LZT (Tc2), we studied B cell deficient (µMT) mice, characterized by a nonsense mutation of the IgM heavy chain resulting in total deletion of B cells. Performing the protocol of sensitization or tolerance induction to contact allergens resulted in robust CHS and LZT in B cell deficient mice in vivo, indicating that B cells are not required for the induction of CHS and LZT. As reported previously, the CHS reaction was slightly impaired in B cells deficient mice as compared to WT mice. Additionally, we analyzed T cell responses in B cell deficient mice after induction of CHS and LZT. Hapten-specific restimulation experiments revealed that lymph node cells from both tolerized WT and B cell deficient mice showed markedly reduced antigen-specific proliferative responses, as compared to LNC derived from sensitized WT and µMT animals. Similar to WT animals T cells obtained from tolerized B cell deficient mice produced typical Tc2 cytokines of LZT with high IL-4 and IL-10 and reduced IL-2 and IFN-? amounts, whereas sensitization of B cell deficient mice resulted in a Tc1 cytokine profile of CHS with high levels of IFN-?. Furthermore, adoptively transferred $CD8^+$ T cells from sensitized (Tc1) or tolerized (Tc2) B cell deficient mice induced significant contact hypersensitivity and tolerance reactions in WT recipients, demonstrating normal development of hapten-specific effector $CD8^+$ T cells of CHS and LZT in the absence of B cells. Our data demonstrate that B cells as APC are not responsible for the generation of hapten-specific effector T cells of contact hypersensitivity or low zone tolerance to contact allergens.

P065

Are they different? Langerhans cells in the steady state versus inflammation.

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Migrating Langerhans cells (LC) may induce immunity or - in the steady state - maintain peripheral tolerance. A contact allergy model was employed to investigate changes in migrating LC in steady state versus inflammation. We applied 2,4,6-trinitro-1-chlorobenzene (TNCB) onto skin to induce migration of LC to lymph nodes. Dendritic cells isolated from skin-draining lymph nodes of TNCB-treated and control mice were compared in phenotype and function. In response to TNCB increased numbers of LC, as determined by anti-Langerin/CD207 staining, appeared in the nodes. Nearly all of the Langerhans cells already expressed CD80 and CD86 in the steady state, the other members if the B7 family were found just on part of the cells. After induction of inflammation more CD40-, B7-H1 (PD-L1)-, and B7-DC (PD-L2)-positive Langerhans cells were found in the lymph nodes. Furthermore, levels of costimulatory molecules on Langerhans cells were slightly enhanced in inflammation. Another B7 family member possibly involved in tolerance induction, ICOS-L, was never detected on LC. Small amounts of IL-12p40 were measured in unstimulated lymph node LC; increased expression was induced by the application of TNCB. When isolated lymph node dendritic cells were further stimulated by LPS- and anti-CD40, both IL-12 p40 and p70 were amplified in most LC. This was more pronounced in the inflammatory situation. The augmented IL-12 production correlated with more IFNg being produced by the lymph node T cells stimulated by anti-CD3/CD28. Thus, phenotypical differences between LC in steady state and inflammation may not be the predominant markers for immunogenic versus tolerogenic LC. However, cytokine profiles differ strongly and may be a decisive factor for the immunogenicity of LC. (Supported by the Austrian Science Fund P-14949.)

P066

Th1 or Th2 cell programming by pro-inflammatory blood dendritic cells upon Toll-like receptor 4(TLR4)- ligation depends on the first six hours of spontaneous maturation

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The immunoregulatory potential of dendritic cells (DCs) is known to critically depend on their state of maturation. Immature DCs are believed to induce tolerance or Th2 cells, while mature DCs can program Th1 cells. Little is known about the functional state of human DCs circulating in blood and their responsiveness to external stimuli. Focussing on a recently described population of pro-inflammatory blood DCs, identified by the mAb M-DC8, we studied their requirements and dynamics of maturation with regard to their responsiveness to external signals and their immunoregulatory function. We show, that independent of additional stimulation and serum factors mAb M-DC8-purified as well as M-DC8+ DCs enriched by negative depletion, undergo profound phenotypical and functional changes upon culture. Within 3 hours the majority of M-DC8+ DCs expressed the maturation marker CD83 and doubled the amount of HLA-DR molecules on their cell surface. When fresh DCs were stimulated with the TLR4-ligand lipopolysaccharide (LPS) or CD40-L they showed a maturation inferior to that of unstimulated cultures and failed to produce IL-12, yet they produced high levels of TNF-a. In contrast, the same stimuli added to DCs after an initial culture period increased DC maturation, IL-12-production and induced an even higher secretion of TNF-a. To test the biological relevance of these findings we initiated cultures of M-DC8+ cells and allogenic cord blood T cells. LPS-stimulation at the beginning of the cocultures induced an increased programming of Th2 cells. In contrast, LPS-stimulation of DCs matured for 6 hours was very effective in the induction of Th1 cells. We here show that human blood DCs, depending on their state of maturation, either induce Th2- or Th1-cells in response to TLR4-ligation. These findings may be relevant for understanding the in vivo situation where immature DCs in the blood seem to be protected from full activation by LPS. Furthermore, short term maturation of DCs after leaving the vasculature may be recquired to ensure LPS-dependent DC-activation that leads to the programming of a protective Th1-dominated immune response.

P067

Evidence for functional expression of Toll-like receptors (TLRs) in human keratinocytes

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Toll-like receptors (TLRs) are important pattern recognition molecules that activate the transcription factor NF-kappa-B, leading to the production of antimicrobial and antiviral cytokines and chemokines. As keratinocytes present the first barrier against exogenous pathogens in human skin, we were interested in the role of TLRs in these cells. We have recently shown that TLR2, but not TLR4, acts as the specific receptor for the S. aureus cell wall components peptidoglycan (PGN) and lipoteichoic acid in an NF-kappa-B-dependent way on human keratinocytes (J Invest Dermatol, 2003, in press). In the present study, we investigated the complete functional expression profile of the 10 known human TLRs in primary human keratinocytes and in the keratinocyte cell line HaCaT. First, the qualitative and quantitative expression of TLR1-10 in RT-/Real Time PCR of primary human keratinocytes and HaCaT cells was analyzed. Both cell types were found to constitutively express TLR 2, 3, 5 and to a lesser extent TLR9. TLR1 could only be found in primary keratinocytes and TLR 4 only in HaCaT cells. In the next step we analyzed the functional relevance of TLR expression in human keratinocytes. After stimulation with various known TLR ligands, the NF-kappa-B activated chemokine IL8 was measured on the protein level. In primary keratinocytes and HaCaT cells the TLR3 ligand poly IC was the most potent stimulator of IL-8 secretion. PGN (TLR2) and flagellin (TLR5) also caused IL-8 secretion, whereas no significant or specific effects were found for LPS, R-848, loxoribine and CpG-DNA. A corresponding pattern was found in an NF-kappa-B translocation assay after ligand stimulation. These studies provide substantial evidence for functional TLR expression in human keratinocytes. Further studies will have to elucidate the role of the functionally expressed TLRs -triggered activation of keratinocytes for normal skin homoeostasis, immunity and skin diseases.

P068

Treatment of B-Cells with Rituximab Promotes Cross Priming of Autologous Cytotoxic T-Cells by Bystander DC

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Rituximab is a genetically engineered chimeric mouse/human monoclonal antibody (mAb) against the human B-cell restricted cell surface antigen CD20 which is currently used for the treatment of B-cell Non Hodgkin Lymphomas. Its effect is due to an induction of apoptosis in B-cells via CD20 signalling, further its Fc domain recruits effector functions to mediate B-cell lysis via complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC). Like this Rituximab is highly effective in depleting B-cells in vivo. Since B-cells are essential to develop autoimmune disorders, Rituximab has been used in a series of clinical trials in diseases like Idiopathic Thrombocytopenic Purpura (ITP) and Lupus. The role of Dendritic Cells (DC) in this antibody treatment regime still remains elusive. DC have been shown to ingest apoptotic cells and cell debris and to cross-present associated antigens on MHC-class I molecules stimulating potent CTL responses. We were prompted to investigate whether Rituximab treatment of B-cells in addition to its direct effects could also promote the induction of an autologous B-cell specific immune response mediated by cytotoxic T-cells (CTL). For this experimental model DC and B-cells were separated from B-CLL patients. After treatment with Rituximab DC readily phagocytosed autologous B-cells. In a coculture with T-cells from peripheral blood they further stimulated CTL to specifically lyse autologous B-cells. Taken together, our results suggest that antibody treatment of B-cells with Rituximab can in addition to direct antibody mediated mechanisms indeed promote the induction of specific $CD8^+$ CTL responses with a long term effect, independent of antibody half life and bioavailability. However, since the decision whether the B-cell becomes an immunogen is hard to predict, it remains to be elucidated if this particular mechanism also undelies the effects of Rituximab in ITP or Lupus treatment.

P069

Cytokines expressed by stimulated epidermal Langerhans cells: An update

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Due to the difficulties in isolating pure preparations of epidermal Langerhans cells (LCs), their production of inflammatory cytokines is still far from clear. We have recently established a protocol for isolating and culturing ultrapure and viable human LCs with the morphologic, phenotypic and functional characteristics of epidermis-resident immature cells. Our Multiplex-PCR analyses demonstrated that unstimulated LCs are characterized by constitutive expression of IL-6, IL-8 and TGF-ß. Stimulation with LPS induced transcription of IL-1ß, TNF-a and of GM-CSF, with transcripts for TGF-ß and GM-CSF in 99% of the CD1a-positive LCs. Data from gene array experiments revealed differential gene expression in LCs that were stimulated by a combination of CD40 ligand and LPS for 4 h. Most interestingly, there was no significant transcription of IL-12, but we found expression of the newly described proinflammatory cytokine IL-27. After stimulation, the gene of the subunit p28 was expressed by LCs and not by stimulated keratinocytes from the same donor. The LCs showed in addition transcripts of IL-15 and IL-18. It will be of interest to determine the contribution of each of these cytokines in innate and adaptive immune responses, and in particular, to study the role of IL-27 in Th1 development.

P070

Granulocyte-Derived Elastase Is Required for Dermal-Epidermal Separation of Human Skin Induced by Autoantibodies from Patients with Bullous Pemphigoid

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Bullous pemphigoid (BP) is an autoimmune subepidermal blistering skin disease associated with IgG autoantibodies against BP autoantigen 180 (BP180) and BP230. While the pathogenic role of autoantibodies to BP230 remains to be elucidated, we recently demonstrated that BP180-specific autoantibodies from BP patients induce leukocyte-mediated subepidermal cleavage in cryosections of human skin. However, the effector mechanisms of blister formation in the human disease have not yet been fully characterized. The aim of the present study was to identify the leukocyte subpopulations and leukocyte proteases instrumental in dermal-epidermal separation in BP. When incubated with cryosections of normal human skin pretreated with IgG from patients with BP, granulocytes, but not peripheral blood mononuclear cells, were recruited to the dermal-epidermal junction and induced subepidermal splits. A combination of broad-range protease inhibitors as well as inhibitors of serine proteases completely abolished dermal-epidermal separation induced by BP autoantibodies. Complete blockade of human leukocyte elastase activity with a specific elastase inhibitor also resulted in suppression of blister formation. These findings strongly suggest that elastase, released from granulocytes, is essential for dermal-epidermal separation in BP patients´ skin.

P071

Intraepidermal T cells expressing the collagen-binding integrin $a_1ß_1$ (VLA-1) are required for development of psoriasis

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Psoriasis is a chronic relapsing skin disorder with suspected autoimmune pathogenesis. Activated T cells seem to be required for disease activity. Trafficking of leukocytes within tissue and thereby generation of an inflammatory response is critically dependent on interactions with extracellular matrix (ECM) proteins such as collagens. We investigated the functional expression of a major collagen-binding receptor: $a_1ß_1$ integrin (VLA-1) in psoriasis.
VLA-1 was exclusively expressed by infiltrating epidermal but not dermal $CD8^+$ and $CD4^+$ T cells in active psoriasis lesions. To determine the *in vivo* relevance of VLA-1 expression on activated T cells in psoriasis we took advantage of a recently described xenotransplantation model with spontaneous development of psoriasis. Uninvolved human skin from patients with psoriasis were transplanted onto AGR mice, deficient in type I and type II interferon receptors in addition to being knockout for recombinant activating gene ($RAG^{-/-}$). Upon engraftment, mice were treated with either anti-a_1-integrin mAb or isotype control antibody. Skin grafts on mice receiving control antibody developed a fully-fledged psoriasis after 6 weeks as expected. Blockade of VLA-1 inhibited psoriasis formation in 6 out of 6 mice (n=3 patients). Histological analysis demonstrated that treatment with anti-VLA-1 mAb resulted in a significant reduction of both the acanthosis ($p=0.001$) and papillomatosis index ($p<0.0001$). A significant, more than two-fold expansion of epidermal and dermal T cells during disease formation was observed in transplanted grafts treated with control antibody. Expression of VLA-1 on expanded epidermal T cells was present in converted psoriatic skin. In contrast, expansion of intraepidermal VLA-1 expressing T cells was completely blocked after anti-VLA-1 treatment.
Our findings suggest a crucial role for intraepidermal VLA-1 expressing T cells in psoriasis and might provide the basis for new strategies in psoriasis treatment focusing on T cell/ECM interactions.

P072

LFA-1 Function on Dendritic Cells is Regulated by Cytip

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The beta2 integrin LFA-1 is important for transendothelial migration of leukocytes as well as for T cell activation during antigen presentation. To determine the relevance of LFA-1 for antigen presentation of DC, we investigated bone marrow-derived DC (bmDC) from CD18-deficient (-/-) mice, which lack all functional beta2 integrins, in vitro. Surprisingly, antigen presentation of bmDC from CD18-/- mice is not impaired as determined by DC-induced T cell activation. T cell proliferation as well as cytokine production of T cells and DC are similar, when CD18 wt or CD18 (-/-) DC were used. To determine why LFA-1 does not seem to be active in this process we studied transendothelial migration and performed binding studies to ICAM-1. Migration of DC was dependent on ICAM, but not on LFA-1 expression. Also LFA-1 expressing mature DC did not bind to ICAM-1, and binding was not inducible by activation. The binding aviditiy of LFA-1 is regulated by the cytosolic proteins Cytohesin-1 and Cytip. In order to be active, Cytohesin-1 aggregates LFA-1 at the plasma membrane, resulting in enhanced LFA- avidity, whereas Cytip complexes Cytohesin-1 and removes the complex from the inner membrane, resulting in LFA-1 inactivation. In activated T cells, Cytohesin-1 is strongly colocalized with LFA-1 in T cells as determined by confocal microscopy, Cytip expression is not detectable and LFA-1 is active in these cells. In contrast, Cytip expression in DC increases during maturation and colocalized Cytip-Cytohesin-1 complexes accumulate in the cytosol, thereby rendering LFA-1 inactive. Thus, LFA-1 function in DC is regulated by Cytip expression.

P073

Autoreactive T helper 1 and 2 cells against desmoglein 1 in patients with pemphigus foliaceus and HLA class II matched healthy individuals

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Pemphigus foliaceus (PF), a severe autoimmune bullous skin disease with subcorneal loss of adhesion is characterized by the auto-antibodies (auto-Ab) against desmoglein 1 (Dsg1), a desmosomal adhesion molecule. The aim of our study was to identify autoreactive T cells against Dsg1 in patients with non-endemic PF and healthy individuals, to investigate a possible HLA class II restriction and to determine their TCR Vß usage. Six PF patients with auto-Ab against Dsg1 and five HLA class II matched healthy individuals were examined. By MACS cytokine secretion assay, Dsg1-responsive T helper (Th) cells were isolated from patients and healthy controls and cloned by limiting dilution. The generated T cell clones (TCC) were characterized with regard to proliferative response and cytokine profile upon in vitro stimulation with Dsg1. Dsg1-reactive Th1 and Th2 cells were detected at similar frequencies in patients (Th1 18.88 ± 15.13 and Th2 12.0 ± 12.94/ 10^5 PBMC) and the control group (Th1 24.09 ± 23.60 and Th2 9.64 ± 9.57/ 10^5 PBMC). In patients (Th1/Th2 ratio 3.29) and controls (Th1/Th2 ratio 1.33) Th1 cells were more predominant. However, in healthy controls exclusively Th2 cells showed proliferative response against Dsg1. All of the Dsg1-specific TCC from both, PF patients (n=39) and healthy individuals (n=9) were only responsive to Dsg1 but not to Dsg3. Cytokine analysis of the established TCC showed that they expressed a stable cytokine pattern i.e. Th1-like TCC secreted IL-2, TNF-a and IFN-? while Th2-like TCC produced IL-2, IL-4 and IL-5. In summary, our data demonstrate that autoreactivity against Dsg1 is not restricted to patients with PF. The observed dichotomy of autoreactive Th1 cells in the patients and Th2 cells in the healthy donors suggests that distinct T cell subsets may be actively involved in the maintenance of tolerance to Dsg1.

P074

Strongly enhanced $a_v ß_3$-Integrin expression and angiogenesis during recurrent episodes of cutaneous DTHR

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The progression of chronic inflammation depends on angiogenesis. Angiogenesis leads to an increase in the cellular traffic from the blood stream to inflammatory tissue sites and is required for chronicity. Vascular cell integrin $a_v ß_3$ is selectively induced during angiogenesis. To better understand mechanisms of chronic inflammation, we investigated the role of $a_v ß_3$-integrin in recurrent episodes of cutaneous DTHR using RGD-peptide that selectively binds $a_v ß_3$-integrin. Mice were sensitized and challenged with trinitrochlorobenzene (TNCB) to induce and elicit DTHR. To investigate T cell independent cutaneous inflammation mice were challenged with phorbol myristate acetate (PMA). 12h after TNCB or PMA challenge animals were injected with [^{18}F]Galacto-RGD or [^{125}I]Gluco-RGD peptide and scanned in vivo with a small animal positron emission tomograph (MADPET) or uptake was determined by autoradiography. In vivo MADPET images confirmed strongly enhanced RGD peptide uptake after a second TNCB- but not after a single TNCB-challenge. Maximum RGD peptide uptake was observed after the 8th TNCB treatment. Only poor $a_v ß_3$-integrin binding was investigated in PMA-callenged mice when compared to TNCB-challenged littermates. RGD-peptide binding decreases after the 8th TNCB-challenge but not after the 8th PMA-challenge. Immunohistochemical staining confirmed ß3 expression on blood vessels. H&E stained sections showed strongly enhanced angiogenesis in chronic antigen-specific T cell mediated cutaneous inflammation but not in chronic PMA-induced antigen independent inflammation. Thus, angiogenesis and $a_v ß_3$-integrin play an essential role in chronic cutaneous DTHR. Our data represent a new model to investigate angiogenesis in chronic antigen specific T cell mediated immune responses such as atopic dermatitis or psoriasis.

P075

Immunology of the human nail apparatus: Is the proximal nail matrix a site of relative immune privilege?

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Although the nail is a frequent target of both infections and autoimmune disease, our knowledge of normal nail immunology is still very poor. Here, we report unexpected immunodermatological findings obtained upon analysis of the normal nail apparatus of an amputated excess finger of a human infant with polydactyly. By immunohistology, we show that the expression of MHC class I-molecules (HLA-A/B/C) is substantially downregulated in the proximal nail matrix (PNM) while the proximal nail fold (PNF) and the distal nail groove show strong HLA-A/B/C expression. Interestingly, NKI/beteb+ cells (melanocytes) also hadno HLA-A/B/C expression in the PNM, while melanocytes in the PNF and distal nail groove were MHC class I+. CD68 and MHC class II double-positive cells (representing activated macrophages) were observed in the dermis underlying the PNF, distal nail groove and nail bed. However, in the dermis adjacent to the PNM, these CD68+ cells were MHC class II? negative, suggesting that these macrophages have a reduced antigen presenting capacity. A high number of CD1a+ cells (Langerhans cells) was seen in nail bed and PNF, which - like their epithelial counterparts - also expressed MHC class II. In contrast, the PNM had only a few CD1a + cells which were MHC class II - negative, and seemed thus functionally impaired. CD4 + and CD8 + cells were very rarely in dermis around the proximal nail matrix but not the proximal nail matrix itself. Furthermore, proximal matrix showed strong immunoreactivity for potent, locally generated immunosuppressants such as TGF-ß1, a-MSH and ACTH. These results suggest that, besides the immunoprivileged anagen hair bulb, the PNM - an evolutionarily older skin appendage compartment than the hair bulb - is yet another, previously unknown intracutaneous site of relative immune privilege, joining the few such sites currently recognized in the mammalian body.

P076

Adherent, mucoid *Pseudomonas aeruginosa* release Pathogen-associated molecules inducing Macrophage Inflammatory Protein-3a in keratinocytes

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Keratinocytes represent the first cellular line in the host defense against microorganisms like *Pseudomonas aeruginosa* (PA). They are capable of producing antimicrobial agents like defensins and releasing leukocyte chemoattractants to fight microbial infection. However, little is known about the molecular interaction of pathogen associated molecules (PAMs) with keratinocytes leading to immune responses. We hypothesize that Macrophage Inflammatory Protein-3a (MIP-3a) released by keratinocytes upon contact with microorganisms, may play an important role in initiating adaptive immune responses by recruiting immature dendritic cells. We investigated whether bacteria release compound PAMs upon contact with skin, which induce MIP-3a in keratinocytes. Initial experiments revealed indeed MIP-3a induction by bacterial PAMs.

To investigate under which conditions bacteria release these PAMs the effect of bacterial culture conditions on the production of MIP-3a-inducing PAMs was analysed. Supernatants of PA cultured under unstressed or stress conditions (low oxygen, starving conditions) were used to investigate the effects on MIP-3a-induction in HaCaT keratinocytes. RNA was isolated and the relative MIP-3a mRNA expression was determined using gene-specific primers in Realtime-PCR.

We found that supernatants of PA cultivated under stress conditions, which led to adherent, mucoid phenotypes, induced expression of MIP-3a, whereas supernatants of unstressed cultures lacked such MIP-3a-inducing PAMs.

Initial experiments to characterize MIP-3a-inducing PAMs by size exclusion chromatography revealed that these PAMs seem to be heterogeneous showing a molecular mass between 200-600 kDa. Moreover, they seem to be different from PAMs inducing antimicrobial peptides which elute from the column in the effluent corresponding to masses >600 kDa. Anion exchange chromatography revealed that MIP-3a-inducing PAMs have a slight anionic charge.

Our investigations confirm that human skin keratinocytes recognise bacterial PAMs and mount an innate defense by production of chemokines like MIP-3a and antimicrobial peptides. Our results indicate that solely stressed adherent bacteria release such MIP-3a-inducing PAMs. These bacterial PAMs are different from those inducing antimicrobial peptides and proteins in keratinocytes.

P077

Role of autoreactive T cells in a mouse model of autoimmune pancreatitis

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Organ-specific autoimmune diseases, such as multiple sclerosis, psoriasis, rheumatoid arthritis and autoimmune diabetes affect 3-5% of the population. These diseases are believed to be mediated through autoreactive IFN-? producing T cells. To investigate the mechanisms leading to the activation of autoreactive T cells we use a mouse model of autoimmune pancreatitis. 415-EL-I-E mice express MHC II I-E exclusively on the exocrine pancreas and T cell reactivity to the MHC class II I-E antigen is associated with T cell receptor containing vß5 chain. Despite their autoreactive potential TCR-vß5^{+} T cells are not clonally deleted in the thymus or in the periphery and about 4% of the CD4^{+} T cells express vß5 in these mice. We found reduced proliferation and decreased IFN-? production of CD4^{+}vß5^{+} T cells compared to control mice. Surprisingly histological analysis of the pancreas showed inflammation and destruction. Toll like receptor 9 Stimulation by i.p. injection of oligo 1668 led to an enrichment of CD4^{+}vß5^{+} and CD8^{+}vß5^{+} T cells but not vß8^{+} T cells in the draining pancreas lymph node. To prevent autoimmune pancreatitis by deletion of autoreactive T cells 415-EL-I-E mice were back-crossed with 107-I-E mice expressing I-E with the normal tissue distribution (on B cells, macrophages, dendritic cells and thymic epithelium). This resulted in a four fold decrease of autoreactive vß5^{+}T cells without preventing pancreas destruction in 415-EL-I-E/107-I-E mice. To exclude the possibility of T-cell independent autoimmunity we back-crossed 415-EL-I-E mice to an immune deficient C57BL/6 Rag$^{-/-}$ background. Flow-cytometric analysis of the lymphatic tissues of 415-EL-I-E /Rag$^{-/-}$ mice showed a lack of T cells. Surprisingly, pancreas destruction in 415-EL-I-E /Rag$^{-/-}$ mice was even enhanced. Therefore we conclude, that autoimmune pancreatitis in this model is mediated by immune cells other than autoreactive T-cells.

P078

Effects of iloprost infusions on serum cytokine, growth factor and soluble adhesion molecule levels in patients with systemic sclerosis

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Iloprost is a stable prostacyclin analogue commonly employed in the treatment of peripheral vascular disease and also used successfully in the treatment of severe Raynaud's phenomenon (RP) associated with systemic sclerosis (SSc). Several mechanisms of action of the drug other than vasodilation and antiplatelet effects have been demonstrated that may be involved in its clinical efficacy.
The aim of the present study was to investigate the long-term influence of Iloprost infusions on disease activity related factors in the time course up to 6 months after therapeutic infusion of Iloprost in patients with SSc-associated RP.
Serum blood samples were collected from 14 patients (all female, average age 54,4 years; one patient with M. Raynaud, 13 patients with limited SSc) before, after therapy and in the time course 1, 2, 3 and 6 months after therapy and examined for endothelin-1 (ET-1), vascular endothelial growth factor (VEGF) and soluble adhesion molecule (sICAM-1, sVCAM-2 and sE-Selectin) using specific enzyme-linked immunoassays.
All patients showed a clinical reduction in severity and number of Raynaud attacks. Despite interindividual variations mean serum levels of sE-Selectin, sVCAM-1, sICAM-1 and ET-1 were reduced after therapy with iloprost. This effect persisted at least for two months (ICAM-1) whereas serum levels of sE-Selectin, VCAM-1 and ET-1 were still reduced at the end of the study period after 6 months. Serum concentration of VEGF were elevated after infusion with iloprost and returned to the initial level 2 months after the last infusion.

These results further explain the known clinical benefit of iloprost infusions in patients with SSc. These effects might be related to changes in serum levels of disease activity related markers. Apparently iloprost is able to modulate these markers and possibly the clinical outcome for a longer period after infusion.

P079

IFN-? is a potent catagen inducer in human anagen hair follicles, likely via induction of TGF-ß2

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IFN-? appears to be an important hair cycle modular in mice, while it is unclear whether it has similar functions in human hair follicles. Therefore, we have studied whether IFN-? can be exploited to modulate the growth, pigmentation and/or cycling of organ-cultured human anagen scalp hair follicles, as an *in vitro*-indicator system for how IFN-? may affect human hair growth *in vivo*. In addition, we wanted to establish a new, simple tool for the rapid experimental induction of catagen *in vitro*. For this purpose, normal human scalp hair follicles in the anagen VI stage of the hair cycle were microdissected cultured according to the Philpott method, with or without IFN-?(75-1000 I.U. mL^{-1}). Hair shaft elongation and pigmentation changes were measured, complemented by quantitative histomorphometry to assess changes in hair follicle cycling (hair cycle score), proliferation (Ki67) and apoptosis (TUNEL). Since TGF-ß2 is a recognized key inducer of catagen in human hair follicles, TGF-ß2 expression was also investigated by tyramide signal amplification and RT-PCR in vehicle- and IFN-?-treated anagen hair follicles. 100-1000 I.U. mL^{-1} IFN-? rapidly and dose-dependently inhibited the hair shaft elongation in cultured human anagen hair follicles and induced morphological signs of catagen transformation after only 4 days of culture, i.e. faster than with all other reported catagen-inducers (e.g. TGF-ß2). Proliferation was significantly inhibited and apoptosis increased in IFN-?-treated hair bulb keratinocyte *in situ*, while follicular melanogenesis appeared unaltered as long as the hair follicles remained in anagen VI. TGF-ß2 immunoreactivity and mRNA transcript levels were enhanced in IFN-?-treated hair follicles. These data suggest that IFN-? is a potent catagen inducer in normal human scalp hair follicles, likely at least in part via up-regulation of the recognized catagen-stimulatory growth factor TGF-ß2. We also show that IFN-? administration offers an excellent tool for experimental catagen induction in organ-cultured human anagen hair follicles.

P080

Heat shock protein 60: The causative antigen for Psoriasis vulgaris?

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Background:
Psoriasis is an inflammatory autoimmune disease of the skin and joints. CD4+ Th1 lymphocytes are suggested to be the main disease-inducing population. But so far, the initiating antigen is unknown. Heat Shock proteins (HSP) are suspected to belong to these antigens that are recognized by the psoriasis plaques infiltrating T lymphocytes.
Methods:
Blood samples and skin biopsies of patients with a history of more than one year of psoriasis were taken. The proliferation of peripheral blood mononuclear cells (PBMC) after stimulation with either HSP60 or Staphylococcal Enterotoxin B (SEB) as positive control was measured. In addition, lymphocytes were isolated from skin biopsies taken of a typical psoriasis plaques. If enough, cells were classified by FACS analysis. The cells were then stimulated with HSP60 or SEB, together with blood-derived dendritic cells as antigen presenting cell, and again the proliferation was measured.
Results:
Blood samples were obtained from 67 patients (43 male, 24 female; age 21 to 84 years) with informed consent . Skin biopsies were acquired from 56 of these patients. Cultured PBMC from 57 and cultured skin cells from 35 patients showed proliferation to SEB as positive control. The proliferation index (proliferation to HSP60 in relation to proliferation to medium) of PBMC of the patients (n=57) was 3.32+/- 5.24 (mean +/- standard deviation). FACS analysis (n=44) of the skin cells showed a pattern with increased levels of CD8+ IFN?+-cells. Of 35 patients which passed quality control, 4 patients (11%) showed a proliferation index of skin cells higher than 2.5 fold.
Conclusions:
Many of the psoriasis patients showed proliferation of PBMC to HSP60. Additionally, about 11% of the patients showed lymphocytes in the psoriasis plaques that could recognize HSP60. So, HSP60 may play a role in the pathogenesis of psoriasis.

P081

Autoantibodies against Laminin 5 are frequently detected in cicatricial pemphigoid, but also in a subgroup of bullous pemphigoid patients by novel Laminin 5 assays.

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The pemphigoid group comprises autoimmune blistering disorders of the skin that are associated with tissue-bound and circulating IgG autoantibodies (auto-ab) directed against components of the epidermal basement membrane zone (BMZ) leading to subepidermal loss of adhesion. Laminin 5 (LN5) is a heterotrimeric multifunctional extracellular matrix glycoprotein of the BMZ. It has been attributed an important role in the initiation and maintenance of epithelial cell anchorage to the underlying connective tissue.
Since BP180 and LN5 are hypothesized to function as natural ligands, LN5 specific auto-ab may contribute to pathogenesis not only in CP, but also in BP. We therefore serotyped a large patient cohort with BP and CP. For this purpose, we developed a novel ELISA and dot blot assay utilizing purified LN5 from squamous carcinoma cell (SCC-25)-conditioned medium, to detect LN5-specific auto-ab in 74 BP, 27 CP and 35 control sera.
8 of 27 (29.6%) CP and 8 of 74 (10,8%) BP sera were LN5-reactive by ELISA. Dot blot analysis revealed that 48.1 % of CP sera and 13.5% of BP recognized LN5. LN5 reactivity of the sera was confirmed by dot blot assay in the majority of BP and CP cases.
The presence of LN5-specific auto-ab is a characteristic feature of CP. In addition, LN5-specific auto-ab are generated also in a subgroup of BP. It needs to be clarified whether these finding is of pathogenetic relevance and/or represents an applicable surrogate marker.
The LN5 ELISA and dot blot assays proofed as sensitive and specific in the detection of LN5 specific IgG auto-ab and, hence, extend the panel of diagnostic tools for bullous autoimmune diseases of the pemphigoid group.

P082

Keratinocytes in early Leishmania major infection: Epidermal production of the Th1 inducing cytokine osteopontin preceds more rapid infiltration of macrophages in resistant mice.

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Cytokines produced at the site of infection may play a pivotal role in controlling early T-helper cell development in Leishmania major (L.m.) infection.We therefore analysed early cutaneous expression of the Th1 inducing cytokine ostepontin (opn) in L.m. infection. Opn mRNA expression was induced as early as 3 h, peaked after 24h and declined to control levels after 3 days. In resistent C57/Bl6 mice opn expression levels were significantly higher compared to susceptible Balb/c mice. In-situ-Hybridisation and immunohistochemistry revealed strong opn mRNA and protein expression in keratinocytes. Induction of opn in the epidermis was confirmed by Northern-Blot analysis of epidermal sheets and immunoprecipitation of extracellular fluid from infected footpads revealed a higher induction of secreted opn in C57/Bl6 mice 24h after infection. Co-culture of keratinocytes and L.m. in vitro was not sufficient to induce detectable opn-secretion suggesting a complex mechanism of opn-induction by L.m.Opn is known to induce IL12 and inhibit IL10 production by macrophages and acts as a potent chemotactic factor for macrophages. We therefore analysed IL12 and IL10 expression in skin and lymphnodes during the first week of L.m. infection in C57/Bl6 and Balb/c mice. Both cytokines were found to be induced during infection. However no differences in IL12 expression between both mice strains could be detected. The induction in of IL10 RNA was even more pronounced in resistant mice. In agreement with these data opn did not affect IL12 and IL10 secretion in isolated tissue macrophages in vitro. In contrast we observed a correlation between higher opn expression and macrophage infiltration in resistant mice as relvealed by immunohistochemistry and detection of F4/80 mRNA by Real-Time PCR. The composition of the early cellular infiltrate is known to affect both T-cell development and outcome of L. major infection. In summary these results identify keratinocytes as a novel player in early experimental leishmaniasis and suggest a crosstalk between keratinocytes and macrophages in the very early phase of infection.

P083

***S. pyogenes*-DNA is not detectable in affected skin or blood of psoriasis patients by PCR**

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Psoriasis vulgaris is an autoimmune disorder of the skin affecting 1-2% of the Western population. In type 1 psoriasis, first onset of disease is associated with streptococcal infection. Immunologic cross reactivity between streptococcal antigens and skin proteins as well as deposition of streptococci both have been discussed as potential explanation for this concurrence. We wondered whether streptococci might be present in psoriatic skin lesions or in the blood of psoriasis patients. Therefore we performed PCR studies with two different primer pairs to amplify streptococcal DNA from samples from psoriasis patients and controls. Genomic DNA was isolated according to standard procedures from 18 lesional psoriatic biopsies, 25 blood specimens, and 23 pharyngeal swabs from psoriasis patients with chronic plaque type psoriasis. Additionally, antistreptolysine-O (ASL-O) and anti-DNaseB (ADNaseB)-titers were assessed serologically. Skin biopsies from patients with dermatoses other than psoriasis were used as controls. One primer pair amplified a specific sequence of the streptolysine-O gene of *S. pyogenes* (length: 633 bp), the other a specific sequence of the MF-gene of *S. pyogenes* (length: 497bp). Amplificates of the second PCR were processed further by Southern blotting and non-radioactive specific hybridization.
Results: The overall frequency of elevated ASL-O resp. ADNaseB-titers was 32%. PCR followed by specific hybridization revealed DNA of *S. pyogenes* in 3 out of 23 pharyngeal swabs and in 2 out of 18 biopsies from psoriatic skin, but not in blood samples or in the control skin samples.
Conclusion: DNA of *S. pyogenes* could not be detected in blood samples, and it was found only in 2 psoriatic skin samples. Here, DNA might origin from superficial colonization by *S. pyogenes*, although bacterial swabs were negative . Therefore, on the level of bacterial DNA there is no definite proof for intracutaneous presence of *S. pyogenes* as target of the psoriatic immune response.

P084

BP180 (BPAG2) and BP230 (BPAG1) harbour epitopes for both autoantibodies and autoreactive T cells in bullous pemphiogoid BP180 (BPAG2) and BP230 (BPAG1) harbour epitopes for both autoantibodies and autoreactive T cells in bullous pemphiogoid

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Although bullous pemphigoid (BP) is a common model of antibody mediated autoimmunity, it is also generally accepted that autoreactive T cells contribute to pathogenesis of BP. The hemidesmosomal adhesion molecules BP180 (BPAG2) and BP230 (BPAG1) are major autoantigens and significantly contribute to dermoepidermal adhesion. Our investigations were directed to characterize autoantibody profiles and autoreactive T cell responses. Using recombinant baculovirus expression of BP180- and BP230-derived proteins, we characterized 50 patients with acute onset BP. All patients developed IgG autoantibodies against the extracellular domain of BP180. The majority of patients, however, displayed IgG reactivity against the noncollagenous NC16A domain of BP180. Several patients, however, had IgG reactivity directed against the C-terminus and other regions within the extracellular part of BP180. In addition, most patients developed autoantibodies against BP230, whereby the C-terminus of the molecule was more frequently targeted than the N-terminus. On this BP collective autoreactive T cell reponses were analyzed. Autoreactive T helper cells preferentially recognized epitopes within the N-terminal region of the the extracellular tail of BP180. Obviously, autoantibodies and autoreactive T cells are targeting similar or identical epitopes within the autoantigen.
Until present, we have identified an immunodominant peptide in the N-terminal region of the extracellular domain of BP180 for which a autoreactive CD4+ T helper clone is specific and restricted by the HLA-DR molecule. Future plans include the induction specific anergy by immune modulation and development of specific immunotherapies in bullous pemphigoid.

P085

Characterization of the skin-derived antimicrobial protein RNase 7

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Human skin is able to mount a fast response against invading microorganisms by the release of various antimicrobial proteins. Recently, we isolated the antimicrobial protein RNase 7 from stratum corneum extracts. To further explore the role of RNase 7 in the chemical defense system of human skin, we performed a detailed analysis of its antimicrobial activity and studied its gene regulation in primary keratinocytes.
For analysis of antimicrobial activity we used RNase 7 isolated from stratum corneum as well as recombinant RNase 7 expressed as a fusion-protein in *E. coli*. RNase 7 exhibits a broad spectrum of high antimicrobial activity against many pathogenic Gram-negative and Gram-positive bacteria including Vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis* and MRSA. Blocking the ribonuclease activity of RNase 7 did not reduce its antimicrobial activity against *E. coli* suggesting that the ribonuclease activity is not essential for antimicrobial activity.
In contrast to the inducible beta-defensins hBD-2 and hBD-3, RNase 7 is expressed in normal skin at a very high level (approximately 100-400 µg RNase 7 were purified from 50 g stratum corneum extracts). Luciferase gene reporter assays demonstrated that the single intron located in the 5'- UTR is required for the high gene expression of RNase 7.
Real-time RT-PCR revealed high constitutive gene expression of RNase 7 in primary keratinocytes which was upregulated in cells grown at higher confluence. This was in concordance with experiments showing an upregulation of RNase 7 gene expression during calcium-induced differentiation of keratinocytes. The high gene expression of RNase 7 in primary keratinocytes was further increased by treatment of the cells with supernatants of adherent cultures of *P. aeruginosa* and *S. aureus* (up to 50-fold induction) and by treatment with 50 ng/ml phorbol-myristate-acetate (100-fold induction). Interestingly, gene induction was nearly completely inhibited by co-treatment with 10^{-7} M all-trans retinoic acid.
In summary, the high expression of RNase 7 in keratinocytes together with its high antimicrobial activity suggests that RNase 7 might play an important role in the innate immunity of human skin.

P086

Early mast cell degranulation controls toxicity of alpha toxin

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Normal protective immunity to bacterial infections requires the induction of early inflammatory responses by activated mast cells (MC). However, little is known about how MC influence the course and maintenance of the following immune reaction. Here, we asked whether MC provide protection against bacterial infection by controlling the toxicity of exotoxins, i.e. alpha toxin (AT) from Staph. aureus. Intradermal injections of AT (0.05-2µg) into the ears of C57BL/6 mice resulted in long lasting and dose dependent inflammatory responses including necrosis at higher concentrations. To test whether AT toxicity is increased in the absence of MC, we injected 0.1µg AT or vehicle into the ears of genetically MC-deficient Kit^{W}/Kit^{W-v}-mice and normal *Kit*+/+ mice. As assessed by measuring ear thickness, Kit^{W}/Kit^{W-v}-mice displayed a significantly stronger inflammatory response (for almost 2 weeks) as compared to ears of normal *Kit*+/+ mice (day 7 after injection: 446±43µm vs. 273±34m, $p<0.01$). To test whether AT toxicity is indeed reduced by skin MC, we injected AT into the ears of Kit^{W}/Kit^{W-v}-mice reconstituted with bone marrow-derived cultured MC. Notably, we found that AT-induced skin damage in adoptively MC-transferred ears of Kit^{W}/Kit^{W-v}-mice was greatly reduced as compared to naive (unreconstituted) Kit^{W}/Kit^{W-v}-ears and virtually identical to that in normal *Kit*+/+ mice. Surprisingly, very early inflammatory responses after injection of AT, a potent MC secretagogue ex vivo, were markedly reduced in the absence of MC (128±8µm vs. 176±7µm, $p<0.01$). MC reconstitution resulted in complete repair of early inflammatory responses in Kit^{W}/Kit^{W-v}-mice indicating that MC may control AT toxicity by promoting very early inflammatory responses, which in turn are induced, at least in part, by AT itself. Our findings suggest that bacterial toxins such as AT are important virulence factors that harm the host, but also function as crucial activating signals for skin MC, triggering more effective immune responses.

P087

C5a modulates phenotype, cytokine production and T cell programming of M-DC8+ human blood dendritic cells

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We previously identified a novel pro-inflammatory type of human blood DCs dendritic cells (DCs) that can be identified by the mAb M-DC8. These cells express receptors for C5a at high cell surface density and were shown to rapidly migrate to the site of C5a challenge in a SCID mouse model. C5a-receptor expression was not observed on other blood DCs (DC1:CD11c+, CD123dim, CD16neg and DC2:CD11c-, CD123high, CD16neg). We now asked for the immunomodulatory effects of C5a on M-DC8+ DCs. Interestingly, rhC5a induced a 4- to 8-fold increase of IL10-secretion; IL6- and TNFa-levels were unaltered. Incubation of M-DC8+ DCs with C5a prior to the stimulation with LPS led to a substancial reduction of the LPS-driven IL12(p70 and p40)-production. Furthermore, cultures of M-DC8+ DCs containing C5a showed lower expression levels of CD80, CD83 and CD40 and these cells had a significantly reduced capacity to promote the proliferation of CD4+ CD45RA+ cord blood T cells. Finally, we studied the Th1/Th2-programming of naïve T cells by M-DC8+ DCs preincubated with C5a. In control cultures we regularly observed a strong bias towards the induction of IFN-g-producing Th1 cells. However, T cell cultures primed with C5a-treated M-DC8+ DCs revealed an increased production of the Th2-cytokine IL-4.
While C5a has been considered as a “complete” pro-inflammatory mediator because it elicits chemotaxis, stimulates smooth muscle contraction, increased blood flow and promotes microvascular permeability, we show here, that C5a-stimulation changes the balance of IL-10- and IL-12-production by M-DC8+ DCs and stimulates the increased programming of IL-4-producing T cells.

P088

Effects of Intravenous Immunoglobulins on Lymphocyte Activation In vitro

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Apart from specific detection of antigens, immunoglobulins are known to modulate a number of cellular and humoral parameters like macrophages, components of the complement system as well as various adhesion molecules. Though the therapeutic effects of intravenous immunoglobulins (IVIG) are well-documented, their mode of action in various, yet pathogenetically distinct diseases is still not known in detail. Therefore, both cellular and humoral parameters were examined in vitro in this study.
Peripheral blood mononuclear lymphocytes from healthy volunteers were separated by gradient centrifugation and stimulated in vitro with phytohemagglutinine (PHA), tetanus toxoid and IL-2. Cell proliferation was studied using a chromogenic assay as well as secretion of both IFN? and IL-4 in specific ELISAs. Stimulation experiments were performed in the absence of IVIG as well as adding serial dilutions of four different preparations within the range of 2.5 mg/ml to 10 ng/ml.
Though stimulation parameters were grossly diverging among the different leukocyte donors, an increase of cell proliferation was seen at the lowest IVIG concentrations of 2.5 mg/ml with subsequent decrease of proliferation rate at higher concentrations. These effects were similar, though quantitatively different in amplitude among the different IVIG preparations. Whereas IFN? secretion upon PHA stimulation was decreased by IVIG, no effects on IL-4 secretion were seen nor on stimulation with tetanus toxoid and IL-2.
In conclusion, this study demonstrates a modulation of leukocyte proliferation by IVIG. The effects were similar for all preparations studied though different in amplitude and may explain their therapeutic response in different diseases. Cellular parameters have to be further studied, however, to explain differential effects in the various target diseases.

P089

Serological diagnosis of pemphigoids: comparison of immunoblotting to ELISA with recombinant NC16a

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Immunoblot analysis using normal human keratinocyte (NHK) extracts is commonly used to detect circulating autoantibodies to collagen XVII/BP180, the major pemphigoid antigen. Recently, the identification of the NC16a domain of collagen XVII as a preferential target of autoantibodies in bullous pemphigoid (BP), pemphigoid gestationis (PG) and -less frequently- in cicatricial pemphigoid (CP) led to the development of ELISA systems utilizing recombinant NC16a.

The aim of this study was to compare the diagnostic value of these immunochemical techniques for the diagnosis of autoimmune subepidermal bullous disorders.

Sera of BP patients (n=17), CP patients (n=5) and PG patients (n=2) were assayed for IgG reactivity by immunoblotting using NHK extracts and by an ELISA utilizing recombinant GST-NC16a fusion protein. The sensitivity of both methods was high since 13 (76%) and 15 (88%) of the BP sera showed positive reactivity with BP180 on immunoblot and ELISA, respectively. Regarding the BP sera, the correlation of both methods was good since 13 sera (76%) reacted equally on WB and ELISA.

Interestingly, three BP sera (18%) showed only anti-NC16a-antibodies by ELISA, while one serum (6%) negative in ELISA had detectable antibodies to collagen XVII by immunoblotting. There were no antibodies to collagen XVII detectable in a BP patient in clinical remission, neither by immunoblotting nor by ELISA.Noteworthy, while none of the 5 CP sera contained detectable levels of anti-NC16a-antibodies by ELISA, one serum (20%) showed a distinct reaction with collagen XVII and four sera (80%) a relatively weak reaction with BP180 by immunoblotting. The two PG (100%) sera were reactive with collagen XVII by both techniques.

In conclusion, the NC16a ELISA is a reliable and very sensitive method in detecting anti-collagen XVII-antibodies in BP and PG patients. The slightly higher sensitivity of the ELISA compared to immunoblotting may be explained by the use of non-denatured proteins. However, if the diagnosis CP is considered, immunoblotting using NHK extracts should be performed in first line.

P090

Expression of human beta defensins 2 and 3 in healthy and diseased human skin

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Defensins are a group of small, cationic, cysteine-rich peptides with broad-spectrum antimicrobial activity and were recently shown to represent an important part of the innate immunity of the skin organ. The purpose of this study was to examine the expression of human ß-defensins (hBD) 2 and 3 in skin samples obtained from different body sites and in inflammatory skin disorders. hBD-2 and hBD-3 mRNA was determined by in situ hybridisation using a specific probe generated in the laboratory on paraffin sections of normal human skin of different localisation and in biopsies from patients with eczema, psoriasis and lichen ruber.

Expression of hBD-2 mRNA was found in only 50 % of the healthy skin samples, especially in areas which are highly exposed to both microbial challenge and UV-radiation (e.g. nose, forehead), whereas hBD-3 mRNA was expressed in all skin samples tested with similar localisation of hybridisation signal most pronounced in the malpighian layer of the epidermis. In addition, in some samples sweat glands were also stained.

In inflammatory skin diseases, increased mRNA expression of hBD-2 was found in eczema, whereas hBD-3 expression was high in psoriasis and only little staining in eczema and lichen ruber lesions.

This study demonstrates the differential expression of hBD-2 and hBD-3 in healthy human skin. Partly absent hBD-2 expression could be explained by the fact, that it is an inducible and not a constitutively expressed antimicrobial peptide. Since hBD-3 is extremely active against Staphylococcus aureus, its lower expression in eczema lesions may explain the clinical experience fact, that atopic in contrast to psoriatic skin is prone to staphylococcus aureus infections.

P091

Mast cells exhibit impaired TLR2/TLR6-dependent stimulation.

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Mast cells (MCs) have been shown to play an important role in innate immunity against pathogens. This view is supported by recent findings showing that MCs express toll like receptors (TLRs) 2, 3, 4, 6, 8, and 9. Furthermore, some bacterial signals, i.e. *Staph. aureus* peptidoglycans (PGN), have been shown to activate MCs via TLR2. Here, we asked whether MCs can be activated by *Mycoplasma*-derived lipoprotein MALP-2, which requires signalling via TLR2/TLR6. While none of the examined MC populations (murine skin MCs, peritoneal MCs, C57 MCs) exhibited degranulation upon stimulation with PGN (via TLR2), LPS (TLR4), or MALP-2 (TLR2/TLR6), activation of MCs via TLR2 or TLR4 resulted in pronounced release of IL-6 and TNFa (IL-6: 1573 ± 328pg/ml [LPS], 1701 ± 1010 [PGN], TNFa: 140 ± 7.2pg/ml [LPS], 341.6 ± 11.5pg/ml [PGN]). However, stimulation of MCs with MALP-2 failed to induce cytokine release from MCs, suggesting impaired functions of TLR2/TLR6 in MCs. When we performed RT-PCR analyses for TLRs and MyD88, MCs were, as previously reported, found to express MyD88, TLR4, TLR6 and the extracellular domain of TLR2. However, MCs failed to express large parts of the TLR2 signalling domain, whereas macrophages showed full length transcripts for TLR2. Our data indicate that MCs express a novel isoform of TLR2 that can not physically associate with TLR6 to exert TLR2/TLR6-mediated effects. Further investigations are required to identify the unique structure of MC-TLR2 and its possible functions.

P092

CD37 is essential for $Fc_{\varepsilon}RI$-mediated mast cell activation.

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CD37 is a member of the tetraspanin superfamily of proteins that have been implicated in cell growth, differentiation, motility, adhesion, and intracellular signaling. Mast cells (MCs) express CD37 and other tetraspanins and stimulation with IgE and antigen leads to upregulation of CD37mRNA. Here, we have characterized the role of CD37 expressed by MCs *in vivo* and *in vitro*. We found that MCs obtained from CD37-/- mice exhibit significantly less degranulation and secretion of TNFa, IL-6, IL-13, and MC protease 1 (MCP-1) after stimulation with IgE+antigen as compared to MCs from wild type mice. To test whether CD37 also contributes to MC activation *in vivo*, we subjected CD37-deficient (CD37-/-) mice and wildtype mice to MC-dependent passive cutaneous anaphylaxis (PCA) or passive systemic anaphylaxis (PSA). Notably, CD37-/- mice showed markedly reduced inflammatory responses in PCA and PSA as compared to wild type mice. These differences are most likely due to impaired MC activation in the absence of CD37 as MCs in CD37-/- mice were found to be less extensively degranulated after PCA as assessed by quantitative histomorphometry. Also, MCP-1 levels after PSA were significantly lower in CD37-/- mice, whereas MC numbers, patterns of distribution, and expression of $Fc_{\varepsilon}RI$ were similar in CD37-/- mice and wild type animals. These results show that CD37 importantly regulates MC activation and MC-dependent inflammatory responses. Our findings also suggest that CD37 may inhibit MC activation by acting as a co-inhibitory membrane adapter protein (rather than by directly stabilising $Fc_{\varepsilon}RI$). Further investigations will have to clarify the mechanism of CD37-dependent inhibition of MC activation.

P093

Expression kinetics of RNA-encoded antigens transfected into DCs

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Dendritic cells (DCs) are considered the most potent antigen presenting cells of the immune system. Several methods have been used to deliver tumor antigens to DCs, such as peptide pulsing, loading with different tumor cell preparations, or viral transfection. Each method has its drawbacks, so new techniques are investigated. Recently, mRNA electroporation (EP) was described as an efficient method to deliver tumor antigens to DCs. Since little is known about the expression of antigens encoded by RNA after transfection into DCs, we studied expression kinetics of several proteins after EP of immature and mature DCs.
More than 90% of the DCs transfected with different amounts of green fluorescence protein (GFP) RNA were positive for GFP. Furthermore, there was a linear correlation between the RNA concentration and the mean fluorescence intensity (MFI) of the transfected cells, indicating that the expression per cell is directly proportional to the RNA concentration. To determine whether the relation of RNA to cell number is relevant, we increased the DC concentration during EP, while keeping the RNA concentration constant. We found that the cell concentration could be doubled without influencing the percentage of positive DCs or MFI. Furthermore, we investigated by intracellular staining if different RNAs, electroporated into the same cell would influence each others expression level. Therefore we used RNA coding for MelanA, MAGE-3 and Survivin, and found no substantial differences in expression levels between one RNA and three RNAs. To study stability and turnover of the RNA-coded proteins we examined the expression kinetics of three different tumor antigens when RNA coding for these antigens was electroporated. In both mature and immature electroporated DCs, expression of these antigens peaked after 3-4 hours. At this time point 80-98% of the DCs were positive. Whereas MelanA and MAGE-3 expression was relatively stable and still near the peak level at 6h, Survivin expression was downregulated faster and protein expression was almost down to background level at 6h. Interestingly, similar antigen expression kinetics were seen in DCs transfected at the mature or immature state.
This knowledge about expression and kinetics can be used to optimize the functionality of DCs in immunotherapy of cancer.

P094

RNA transfection of dendritic cells: advantages of electroporating DCs at the mature stage

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Using the patients own immune system to fight cancer is a promising approach. The cells that are best equipped to initiate an immune response are dendritic cells (DCs) since they express a variety of costimulatory surface molecules and are capable of activating naïve cytotoxic T-cells. For CTL induction DCs must present antigens via the MHC class I pathway that usually presents peptides derived from cytoplasmic proteins. One approach to gain access to this pathway is RNA electroporation of DCs. This method was shown to lead to specific activation of CTLs. Whether mature or immature DCs are a better subject for electroporation is not answered yet. Here we compare the functionality of monocyte-derived DCs that were electroporated at the immature stage and then matured with DCs that were electroporated after maturation. We show that DCs, that were matured before EP displayed smaller alterations in size and granularity. In MLR, mature electroporated DCs stimulated almost as well as untransfected cells, while DCs electroparated at the immature state displayed a clearly reduced potency to activate resting T cells due to electroporation. To determine the antigen specific stimulatory capacity of electroporated DCs, we compared the capability of HLA-A1+ DCs transfected at the immature or mature state with defined RNA for Mage-3 to stimulate a Mage-3-specific HLA-A1 restricted T-cell-clone. We found that mature electroporated DCs were much better stimulators of Mage-3 specific T cells. Since RNA-electroporation results in very short expression of antigen, the stability of the MHC-peptide complexes is important for prolonged antigen-presentation. We show that the half-life of this complex is much higher on mature DCs than on immature DCs. In aggregate, the data on DC functionality suggest that immunotherapy using RNA transfected DCs should be more efficient if DCs are electroporated at the mature stage.

P095

Efficient G-CSF-dependent mobilization of M-DC8+ blood dendritic cells that retain their pro-inflammatory and Th1-programming capacity

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The efficient capacity of dendritic cells (DCs) to initiate anti-tumor immunity has led to clinical studies employing DCs for the immunotherapy of cancer. A limiting factor for using native human blood DCs instead of in vitro generated DCs, is the low frequency of DCs in blood and hence the difficulty to isolate sufficient numbers of DCs. Focussing on the largest human blood DC population which can be directly isolated by a one-step magnetic cell sort using the mAb M-DC8, we studied an approach to increase the frequency of M-DC8+ DCs in peripheral blood and monitored their function. Blood was collected from young healthy adults undergoing G-CSF-stimulation to mobilize hematopoetic stem cells for allogenic stem cell transplantation. During 5 days of s.c. G-CSF-stimulation, the frequency of M-DC8+ DCs in the blood significantly increased in all 13 individual donors tested. The mean frequency of M-DC8+ DCs increased from $21.7x10^6$/L (SEM±3.7) to $69.4x10^6$/L (SEM±14.4). While, as reported by others, the population of plasmacytoid DCs was also expanded, their frequency in blood remained low. Mobilized M-DC8+ DCs preserved the expression levels of MHC class II, CD86, CD40, CD83, CD33 and CD16. Furthermore, comparing M-DC8+ DCs from the same donor before and after G-CSF-stimulation revealed no change in the production of TNF-a and IL-12 upon stimulation of PBMC with LPS or LPS and IFN-? as studied by intracellular cytokine-staining. To ensure a comparison of the T cell stimulatory capacity of M-DC8+ DCs prepared before and after G-CSF-stimulation, the DCs were cocultured at each occasion with an alliquot collected from the identical cord blood T cell sample (CD45RA+, CD4+), kept frozen until usage. In these experiments mobilized M-DC8+ DCs demonstrated an undiminished capacity to prime the proliferation of naïve T cells. Most importantly, M-DC8+ DCs mobilized by G-CSF retained their strong capacity to program Th1 T-cells, which would be profitable for vaccination protocols.
Therefore, G-CSF-mobilization of M-DC8+ DCs might offer a means by which sufficient numbers of highly functional, Th1-inducing dendritic cells can be easily attained for immunotherapy.

P096

In vitro immunomodulatory effects of iloprost in patients with systemic sclerosis

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Iloprost, a stable prostacyclin analogue, has recently been introduced in the treatment of Raynaud's phenomenon associated with systemic sclerosis (SSc). Besides effects on microvascular blood circulation as well as platelet aggregation, immunomodulatory effects have been described. In previous studies we could demonstrate age-dependent effects of iloprost on the proliferation of peripheral blood mononuclear cells (PBMC) of healthy individuals.
In this study the immunomodulatory activity of iloprost on PBMC obtained from patients with systemic sclerosis was studie. PBMC of 11 patients with systemic sclerosis (9 female, 2 male; average age: 60,2 years; one patient with diffuse SSc, 10 patients with limited SSc) were incubated with PHA, IL-2 and tetanus toxoid (TT). The concentrations of iloprost corresponded to plasma levels obtained after infusion of a therapeutic dosage. Proliferation of PBMC was evaluated in a BrdU-ELISA and cytokine production of both IFN? and IL-4 in specific ELISA formats. Stimulation with 50 ng/ml iloprost induced an increased proliferation of PBMC in 6/11 patients, 2 showed a decreased proliferation and 2 were unchanged. Upon 150 ng/ml iloprost, 10 patients showed a decreased proliferation whereas in 1 proliferation remained unchanged. Similar, although not as distinct effects could be seen upon stimulation with TT and IL-2.
After clinical intravenous application of iloprost, the mean values of PBMC proliferation obtained from the same patients revealed reduced rates upon PHA and no changes when stimulated by TT and PHA. Compared to the mean values of a control group (11 healthy females; average age: 54,4 years) differences were seen with TT (control showed lower values) and IL-2 (control showed higher values). In contrast, iloprost had no influence on the cytokine production. Whereas IFN? production in PHA stimulated cultures was always high and could not be modulated by iloprost, Il-4 was always below the detection limit of the ELISA.
The data on cell proliferation indicate immunomodulatory effects of iloprost in patients with systemic sclerosis. These effects might add to its clinical benefit seen in systemic sclerosis and possibly other immunomodulated diseases.

P097

Identification of novel genes regulated by alpha-melanocyte-stimulating hormone in murine bone marrow-derived dendritic cells

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Many strains of evidence indicate that alpha-melanocyte-stimulating hormone (alpha-MSH) elicits its immunomodulatory activity via binding to melanocortin receptors (MC-Rs) expressed on monocytes and dendritic cells. In order to identify novel target genes regulated by alpha-MSH in these cells, we prepared bone marrow-derived dendritic cell precursors from Balb/c mice and treated them with GM-CSF and IL-4 for 6 days. The MC-R profile on these immature dendritic cells was first determined by quantitative RT-PCR. Both transcripts for MC-1R and MC-5R were detected in these cells. Cells were subsequently stimulated with dinitrobenzene sulfonic acid (DNBS), alpha-MSH or both substances for 2 or 16 hours. After RNA preparation, cDNA synthesis and in vitro transcripton hybridization of biotinylated cRNA samples was performed on MG U74A Affymetrix gene chips. Data evaluation, cleansing, extraction and analysis of the more than 12 000 cloned genes and expressed sequence tags were performed using the Gene Data Analyst vs.1 Expressionist software. Filter criteria included a minimum threshold of 100, normalization by the logarithmic mean and a quality setting of $p<0,04$. Changes with a change factor of >2 were regarded as significant. As expected, stimulation with DNBS resulted in induction or upregulation of genes encoding proinflammatory cytokines, growth factors, signal transduction intermediates and transcription factors. Treatment with alpha-MSH blocked the DNBS-driven upregulation of several known genes such as IL-1 or CD86. On the other hand, alpha-MSH modulated the expression of several novel genes implicated in immunomodulation, e. g. IL-1beta converting enzyme, IFN-gamma receptor, FK506 binding proteins or several neuropeptides and their receptors. These data indicate novel molecular targets by which alpha-MSH exerts its immunomodulatory activities in immunocompetent cells.

P098

Reproducible induction of tumor-specific cytototoxic and helper T cells in melanoma patients does not require CD40L stimulation or cognate T cell help when mature monocyte-derived Dendritic Cells are used as a vaccine

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We are interested to systematically optimize vaccination with monocyte-derived (Mo-) dendritic cells (DC) by adressing important variables in two-armed trials with immunogenicity as an important end-point. To investigate the paradigm, whether the induction of class I restricted CTL responses by DC requires the presence of class II restricted T cell helper epitopes on the same DC or alternatively CD40L stimulation of the DC, we vaccinated stage III and stage IV HLA-A2.1+ and/or HLA-A1+ melanoma patients with mature Mo-DC pulsed with MHC class I peptides (pulsed onto different DC batches to avoid peptide competition) for a given HLA molecule plus or minus CD40L. Unspecific helper proteins (e.g. TT or KLH) were avoided as they blur the interpretation of induced immune effects. Select tumor-specific MHC class II restricted helper peptides were pulsed onto DC batches distinct from the DC pulsed with the MHC class I peptides. Extensive serial immunomonitoring revealed in most patients a rapid induction of T cells specific for most MHC I and II restricted tumor peptides used. The responses were not enhanced if the respective peptides were delivered on DC pretreated with trimeric CD40L. These results demonstrate, quite surprisingly, that mature Mo-DC can induce helper T cells but also a robust priming of MHC class I restricted T cell responses without any cognate T cell help or CD40 triggering. While these results are certainly encouraging, we are now also in a position to scientifically test the effect of including different types of cognate T cell help or respective mimicks on ensuing T cell responses, notably the generation of effective T cell memory which will be decisive for a successful cancer DC vaccine.

P099

Large scale generation of mature dendritic cells from monocytes enriched by elutriation

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Dendritic cells (DC) are promising tools for the immunotherapy of cancer. The induction of tumor-specific T cells and concomittant clinical regressions have been observed in early phase I/II trials by using either DC isolated directly from blood, DC generated from CD34+ precursors ex vivo, or most frequently by employing monocyte-derived DC. As DC-vaccination is now awaiting phase II/III trials with larger patient collectives it becomes increasingly important to obtain large numbers of cells suitable for therapeutic application under labor- and cost-effective conditions.
We describe here a procedure that uses a modified cell seperator (CES; Cell Elutriation System, Gambro BCT) to enrich monocytes from a whole apheresis product within one hour. Cells are separated on the basis of size and, to a lesser extend, density by counterflow elutriation in a novel 40 ml conical chamber. The mean monocyte recovery following elutriation (n= 6) was 82,3 % (36,7 % - 100 %)), the mean purity 60,97 % (48,09% - 71,73%). By using GM-CSF + IL-4 followed by a standard maturation cocktail composed of IL-1ß IL-6, TNF-a and PGE_2 these monocytes could be differentiated into fully mature dendritic cells suitable for successful cryopreservation.
Enrichment of monocytes for subsequent DC-generation with CES is a fast, easy to perform method in a closed system. DC are phenotypically and functionally mature and therefore applicable for immunotherapeutical approaches. The method circumvents a density gradient step to enrich PBL from leucapheresis products and does not require (xenogeneic) antibodies to target monocytes. At present, limitations are a considerable red blood cell contamination which is currently addressed in ongoing experimental runs.

P100

Antigen specific immune responses of melanoma patients following vaccination with peptide loaded dendritic cells as second line therapy are indicative of a prolonged survival

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6 pretreated stage IV melanoma patients (pts) with progressive disease were repeatedly vaccinated intracutaneously with peptides-pulsed dendritic cells (DC) at 2-4 weeks interval and, after 3 months immunization, clinical and immunological responses analyzed. DC were generated by culture of monocytes, isolated from leukapheresis products, in medium containing GM-CSF and IL-4 and differentiated into mature DC via addition of IL-1ß, IL-6, TNFa and prostaglandin E_2 on day 6. Mature DC were pulsed on day 7 with HLA-matched peptides of tyrosinase, gp100, MAGE-3, and MAGE-1 (60µg/ml, $3\text{-}5x10^6$ DC per peptide) for 2 hours. Immune responses were measured by IFN-? Elispot assay detecting peptide specific CD8+ T cells without prestimulation or addition of cytokines.
1/6 pts with metastases of lung and lymphnodes responded with a complete remission (CR) after 10 vaccinations. He still is in CR after 2 years. 1/6 pts showed progression during vaccination alone, which could be slowed down by combining the vaccination approach with DTIC chemotherapy. The overall survival was 19 months after start of the combined therapy. In these 2 pts positive immune responses could be detected showing an increase of the median number of specific T cells from 1<100,000 to 1/7518 and 1/2857, respectively. 4/6 pts demonstrated progressive disease with overall survival of only 4-6 months. In these pts no specific immune responses to any of the vaccinated peptides loaded on DC were detectable.
In conclusion even advanced melanoma pts may benefit from tumor specific vaccination with peptid-pulsed DC if they are capable to develop a specific immune response. In the case of tumor progression a combined immunochemotherapy should be considered.

P101

New tools to monitor T cell responses following repeated vaccinations with peptide-loaded dendritic cells: in vivo migration to regional lymph nodes and rate of apoptotic DC at local injection sites

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P102

Control of tumor angiogenesis by adaptive immunity through TNF receptor 1-signaling

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The transgenic mouse model RIP1-Tag2 is an established model for studying multistage tumorigenesis and angiogenesis. In these mice the large T antigen (Tag) of the Simian Virus 40 is only expressed in insulin-producing ß cells of the pancreas, leading to the successive development of islet cell hyperplasia, adenomas, and finally invasive, highly vascularized carcinomas. We have previously shown, that tumor-specific Th1 cells can delay tumor growth in RIP1-Tag2 mice. We investigated, whether Tumor necrosis factor (TNF) and TNF receptor 1 (TNFR1) signaling play a mayor role in tumorigenesis.
At 6-7 weeks, either RIP1-Tag2 or double transgenic RIP1-Tag2xTNFR1$^{-/-}$ mice were treated with Tag specific Th1 cells weekly. We followed, blood glucose, histology and angiogenesis. Therapy with Tag-specific Th1 cells prolonged life two-fold in RIP1-Tag2 mice. Double transgenic RIP1-Tag2xTNFR1$^{-/-}$ mice developed carcinomas later then single transgenic RIP1-Tag2 animals, showing that signaling through TNFR1 promotes tumor progression. Surprisingly, adoptive transfer of Th1 cells in RIP1-Tag2xTNFR1$^{-/-}$ mice did not delay tumor growth. In contrast it accelerated the decrease of blood glucose and tumor development when compared to RIP1-Tag2xTNFR1$^{+/+}$ littermates. Moreover, Tag-Th1 cells promote the activation of avß3 integrin in RIP1-Tag2xTNFR$^{-/-}$ mice.
Thus, adoptive transfer of tumor-specific Th1 cells is very effective in preventing the transition of preneoplastic dysplasias into carcinomas. This inhibition seems to be independent of tumor cell killing but strictly dependent on interferon ? (data not shown) and TNF-signaling.

P103

Dimethylfumarate selectively targets CD8-positive T-cells *in vitro*

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Fumaric acid esters are well known for their efficacy in the treatment of psoriasis vulgaris.
Recently, our group was able to show that dimethylfumarate (DMF), the main ingredient of the marketed mixture, is a potent inducer of apoptosis in human T-cells. It is further known that DMF inhibits NF-?B-dependent pathways of cellular activation and secretion of mediators and cytokines.
The purpose of the study presented was to further analyse the effect of DMF on T-cell subpopulations. For this purpose, we seperated CD4-positive and CD8-positive T-cells by magnetic-bead selection and incubated the cells with different concentrations of DMF.
The results confirmed the induction of apoptosis in the two T-cell subsets. Interestingly, we were able to show a dose-dependent decrease in the expression of CD8, not, however, of CD4 by DMF-treatment in comparison to medium alone as determined by flow-cytometry. Expression of the T-cell co-stimulatory molecule CD 28 on CD8-positive T-cells after stimulation with anti-CD3-antibodies (OKT3) or phytohemagglutinine A was decreased in the presence of different concentrations of DMF.
Our results provide evidence that among the known pharmacological mechanisms concerning the anti-psoriatic effects of DMF a selective decrease of CD8-positive T-cells and of surface receptors on these cells is important. Since the inflammatory infiltrate of CD8-positive T-cells in the dermis of psoriatic lesions is regarded to maintain the disease process this observation may be of importance regarding the clinical effect of DMF in chronic plaque-type psoriasis.

P104

Interleukin 5 gene expression in human T lymphocytes via a GATA/t-bet element in position - 150 bp of the human IL-5 promoter

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Cytokine interleukin (IL)-5, produced in large amounts by activated T helper (Th) 2 lymphocytes, plays a pivotal role in the outgrowth of eosinophils and eosinophil tissue infiltration in patients with atopic eczema or allergic bronchial asthma. Transcription of the IL-5 gene is inducible in Th2 but not in activated Th1 cells. Several regulatory elements and trans-acting factors involved in the regulation of Th-specific cytokine genes have been reported. Little is known about the transcriptional regulation of the human IL-5 gene in peripheral T cells. In order to study the molecular regulation of the human IL-5 gene a 774 bp long fragment D1 was isolated from the human genomic lgt11 library, mapped, subcloned and sequenced. To delineate the molecular regulation 6 different deletion fragments from -507 to ?43 bp of the 5´upstream region were generated and fused to the CAT gene. In transient transfection experiments with the human Jurkat cell line. Upon transient transfection of the indicated IL-5 promoter-CAT constructs, only the plasmid pJL5 (-57 to +43 bp) showed low constitutive reporter gene activity 20 h after electroporation. Deletion of the enhancer/promoter region spanning from -505 to -157 bp, according to the transcriptional start site, did not lead to stronger CAT activity. Interestingly, no specific IL-5 mRNA or protein was detectable in resting or stimulated Jurkat cells. Deletion between -157 and -57 bp resulted in a 7-fold increased basal induction, pointing to a negative regulatory element within this region. Gel shifts with competition analyses using 9 truncated or mutated dsDNA fragments spanning -138 to -157 bp of the human IL-5 promoter were applied to further delinate critical bases for transcription factor binding. A slower migrating specificity of the dsDNA oligo is depending on adenosine residues in position -156 and -155 bp and an neighboring intact GATA-motif (TTAAAAGATAAAAGTAAATT). This leads to suspect a negatively regulating motif, involving the transcription factors GATA and t-bet in the human Jurkat cell line. Physiological control of IL-5 gene expression in unactivated and stimulated clonal Th1 cells could be explained by this mechanism as well.

P105

Determination of coronary artery calcium in patients with psoriasis: Prospective trial to detect the incidence of cardiovascular disease

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Early reports from retrospective studies performed in the 70's showed an increased incidence of cardiovascular disease in patients with psoriasis. However, while some follow-up studies confirmed this data, others could not document this finding. Hence, the association of cardiovascular disease with psoriasis remains an uncertain issue. In addition no prospective trials have been performed, and other risk factors for cardiovascular diseases have not been taken into account in the performed studies. However, experimental data have clearly documented a procoagulant state in patients with psoriasis, supporting the association of cardiovascular disease with psoriasis.
We therefore initiated a prospective study to measure the level of coronary artery calcium using multi-slice computer tomography, a non-invasive method for detection of coronary artery disease. Patients with an at least 10-year history of psoriasis and a documented server disease were included. Patients with a history of cardiovascular disease were excluded. In addition, risk factors for cardiovascular disease were assessed.
Upon submission of the abstract 10 eligible patients have been included. So far no difference can be detected in patients compared to published data on coronary artery calcium in controls: Age group 40-49 (n=6): 3±3 vs. 27±9; age 50-59 (n=3): 81±55 vs. 83±14; age 60-69 (n=1): 161 vs. 187±38. Due to the low number of patients included and comparison to published, rather than own controls, final conclusions may not be drawn from this data. We plan to include at least 50 patients and 50 age-, sex- and risk factor-matched controls before final evaluation of the data.
In conclusion, due to the inapt control group and the low number of patients included, the association of cardiovascular disease with psoriasis remains uncertain. However, the prospective determination of coronary artery calcium in patients compared to an appropriate control group may help to determine if patients with psoriasis are at risk for cardiovascular disease.

P106

Pyruvate kinase type M2 is a predictive marker in malignant melanoma

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Due to their enhanced rate of proliferation, malignant cells are strongly dependent on a sufficient energy supply by the enzymatic cascade of glycolysis. To provide these high amounts of energy, the key enzyme of the glycolysis, the pyruvate kinase (PK), is expressed as a type M2 isoform (M2-PK) by neoplastic cells. M2-PK molecules are released into the peripheral blood and may hereby function as a potential marker predicting tumor load and prognosis of cancer patients. In the present study we measured the concentration of M2-PK in plasma samples from 300 melanoma patients and 53 healthy controls using ELISA technique. The median plasma concentration of M2-PK was significantly elevated in patients versus controls (9.30 U/ml versus 7.20 U/ml; $p = 0.0036$). This increase of plasma M2-PK in melanoma patients was found to be strongly dependent on the stage of disease ($p < 0.00001$) and the tumor load ($p < 0.0005$). In regard to the prognosis, patients with an elevated plasma concentration of M2-PK (cut-off = 15 U/ml) showed a strongly reduced overall ($p < 0.000005$) and progression-free ($p = 0.023$) survival compared to patients with normal M2-PK plasma values. Our results indicate that the plasma concentration of M2-PK may function as a reliable and useful marker for the early detection of a tumor relapse as well as for the prediction of the prognosis in melanoma patients. Further studies comparing M2-PK with conventional predictive markers like S100-beta will be needed to prove the clinical value of this new marker.

P107

Image enhancement using mathematical morphology in in vivo laser scanning microscopy of the skin

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In vivo confocal laser scanning microscopy (CLSM) is a new method that provides skin images in horizontal plane at a level of resolution that allows to view microanatomic structures. In particular, individual cells of the horny layer, the living epidermis, melanocytes and dermal papillae including dermal capillaries can be discerned. This study examinines wether certain digital image processing steps using mathematical morphology can increase the visability of certain structures in CLSM.
Using a Vivascope 1000 system, 50 images were taken from normal skin of 25 probands, and 39 image-enhancement procedures were created. 8 procedures which seemed to provide some quality enhancement, were arbitrarily selected for further evaluation. Subsequently, a collection of random pairs of the original image and an image submitted to any of the 8 selected procedures was rated by 5 independent observers.
In 3 of the 8 procedures tested, the modified image was significantly prefered to the original image (chi-test.: p ? 0.001). In particular, smoothing, shading correction and delineate in various combinations were helpful in showing the characteristic honeycomb- pattern, pigmented basal cell layer, cell borders, and the nuclei more clearly. The best method consisted of the production of an average-filtered image with a large mask size of 49 pixels, which was subsequently reduced in contrast and used as a reference image for shading correction of the original image. Finally, the resulting image was submitted to grey level contrast enhancement with clipping of 1 % of the extreme grey values. Using this procedure, the modified image provided advanced visual interpretation in 80 % (200 out of 250) of the rated image pairs.
Our study shows that digital image processing using mathematical morphology may help to increase visability in in vivo CLSM and may provide superior prerequisites for diagnostic applications.

P108

Comparative Time Course Of Skin Photosensitization And Psoralen Plasma Levels After Systemic Administration Of 5-Methoxypsoralen Or 8-Methoxypsoralen

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In the present study we investigated the duration and magnitude of skin photosensitivity after oral administration of 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP). In addition, psoralen plasma concentrations were determined and correlated with the minimal phototoxicity dose (MPD).
30 healthy volunteers without history of abnormal sun reactivity were included in the study. 15 volunteers each received either 5-MOP (1.2 mg/kg) or 8-MOP (0.6 mg/kg) with a standardized breakfast. MPD testing and blood sampling were performed 1, 2, 3, 4, 6 and 8 hours after psoralen intake. Psoralen plasma levels were determined by high pressure liquid chromatography (lower limit of detection: 40 ng/ml).
5-MOP: Maximum plasma levels with a median of 324 ng/ml (range, 156 - 548 ng/ml) were detected at 2h after drug ingestion. In 8 out of 15 subjects (53%) maximum photosensitivity occurred synchronously with the peak of the psoralen plasma level. The median MPD was lowest at 2h after 5-MOP intake with a median of 2,8 J/cm² (range, 1,4 - 8,0 J/cm²). 8h after administration the median 5-MOP concentration was 47 ng/ml (range, 40 - 120 ng/ml) and the median MPD was 11,3 J/cm² (range, 8,0 - 32,0 J/cm²), respectively.
8-MOP: Maximum plasma levels with a median of 262 ng/ml (range, <40,0 - 441 ng/ml) were detected at 1h after drug ingestion. As with 5-MOP, the time of maximum photosensitivity paralleled the peak psoralen plasma level in 8 out of 15 subjects (53%). The MPD was lowest at 2h after 8-MOP intake with a median of 2,0 J/cm² (range, 1,0 - 5,7 J/cm²). 8h after administration the median 8-MOP plasma concentration was <40 ng/ml (range, 0 - <40 ng/ml) and the median MPD was 16,0 J/cm² (range, 5,7 - >32,0 J/cm²), respectively.
Our study shows that 5-MOP is eliminated from the serum at a rate comparable to that of 8-MOP and that considerable photosensitivity is maintained as long as up to 8 hours after systemic administration of either one of the two psoralen compounds.

P109

Circulating CD4+ T cells in Sezary syndrome express CCR7 as well as CCR10 and may represent a malignant expansion of cutaneous central memory T cells

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Adhesion molecules and chemokine receptors are involved in tissue-specific homing of T cells to the skin. In the present study we investigated the expression of the cutaneous lymphocyte antigen (CLA) and the CC chemokine receptors (CCR) 4 and CCR10 on CD4+ T cells in the peripheral blood of patients with cutaneous T cell lymphoma (CTCL), a malignancy of cutaneous memory T cells. As expected, we found that both CLA and CCR4 are expressed on almost all circulating CD4+ memory T cells. Most of these circulating CLA+, CCR4+, CD4+ memory T cells also express the skin homing chemokine receptor CCR10. We hypothesized that the large accumulation of cutaneous memory CD4+ T cells in the peripheral blood of patients with Sezary Syndrome, a rare leukemic variant of CTCL, might be due to expression of CCR7, a lymph node homing chemokine receptor. Our data indeed show that a significant percentage of circulating clonal CLA+, CD4+ memory T cells from patients with Sezary Syndrome but not from patients with mycosis fungoides express CCR7. These results provide first evidence for the concept that Sezary Syndrome represents a malignant expansion of cutaneous central memory T cells.

P110

Vitamin-D deficiency in sun deprived risk groups including organ transplant recipients under immunosuppressants, patients with xeroderma pigmentosum or basal cell nevus syndrome: What are the consequences?

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UV-exposition is the the main reason for the development of skin cancer. However, 90% of all requisite Vitamin-D is formed within the skin through the action of the sun - a real dilemma, for a connection between Vitamin-D deficiency and various types of cancer (e.g. colon-, prostate- and breast cancer) has been confirmed in a multitude of epidemiologic studies. The cancer protective effect is contributed to the extra renal, local production of 1a,25(OH)2D3, that has been shown in various tissues. We have now screened different sun deprived risk groups (A: patients with genodermatoses connected with defects in sun-induced DNA repair, and B: non vitamin-D substituted renal transplant recipients under immunosuppressants) for their vitamin-D status. As measure of the vitamin-D store and as substrate for the 25-hydroxyvitamin D-1a-hydroxylase basal 25(OH)D3 serum levels (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) have been analysed. Mean basal 25(OH)D3 serum values of 9.5 ng/ml (normal range: 15.0 90.0 ng/ml) in group A (n = 4: 3 patients with xeroderma pigmentosum, 1 patient with basal cell nevus syndrome) and 13,9 ng/ml in group B (n = 33) were measured. So in both groups decreased basal 25(OH)D3 serum levels were detected. In our pilot study we point out that vitamin-D deficiency is a manifest problem in patient groups practising sun protection. In preceding studies a more or less pronounced decrease of basal 25(OH)D3 serum level has been detected in sunscreen users, which seems to have only a minor influence in calcium homeostasis and bone integrity. However, in the light of the novel functions of vitamin-D, especially of the locally produced 1a,25(OH)2D3, a monitoring of the vitamin-D status in sun deprived risk groups should be demanded. Regarding the recently postulated dependence of the local generation of 1a,25(OH)2D3 on the 25(OH)D3 serum level, in case of a decreased or maybe even low normal basal 25(OH)D3 serum value an oral substitution should be recommended.

P111

The Farnsworth panel D 15 test - a simple instrument to detect high risk melanoma patients? First results of a study including 300 patients with malignant melanoma

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Melanoma-associated retinopathy (MAR) is a paraneoplastic syndrome in patients with malignant melanoma, that is rarely seen with all its clinical signs and symptoms. The pathomechanism is supposed to result from antibody production against melanoma antigens which cross-react with retinal epitopes. Beside light sensations, night blindness and visual loss, color loss for blue (tritan dysfunction) has been reported. The onset of MAR symptoms is correlated with a rapid progress of disease and a worse prognosis for progress-free and overall survival. Recently performed ophthalomological examinations as well as immunofluorescence assays on retinal tissue using autologous serum samples of melanoma patients could prove, that subclinical signs as well as the presence of antiretinal antibodies is more frequent than supposed. As intensive ophthalmological examinations and indirect immunofluorescence on retinal slides are time-consuming and cost-intensive they usually cannot be performed in melanoma aftercare. The Farnsworth panel D 15 test is a color test which is able to detect all kinds of color deficiencies. Patients have to arrange 15 different color caps using a blue reference cap. Mapping of the color sequence in a diagram shows the existence of color loss. This study including 100 melanoma patients (AJCC stage 0: n=3; stage I: n=149; stage II: n=53; stage III: n= 56; stage IV: n= 39) and 100 healthy controls was performed to answer the question if the Farnsworth panel D 15 test alone is able to detect high risk melanoma patients. 26% of all melanoma patients showed tritan dysfunction, this percentage was much higher than in the control group (2%). The presence of tritan dysfunction correlated with stage of disease, tumor thickness, Clark Level and age, but not with sex, S-100 value or time since first diagnosis. Actually, follow-up examinations are performed and compared with immunofluorescence experiments with autologous serum on retinal tissue. After this evaluation is should be possible to state if the Farnsworth panel D 15 test is a simple instrument to detect high risk melanoma patients in melanoma aftercare.

P112

Preclinical validation of L-ornithine for topical application

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L-ornithine is a non-essential, non-proteinogenic cationic amino acid. Its complex metabolic function causes a great interest in its pharmacotherapeutical potentials. L-orntihine is critical for the metabolism of keratinocytes, especially in synthesis of urea, polyamines and precursors of collagene. In this study we investigate *in vitro* the cytotoxicity of L?ornithine and its influence of urea-synthesis and the expression of arginase and ornithine decarboxylase in native human keratinocytes. Further investigations evaluate the possibility of topical dermal application of L-ornithine and its *in vivo*-toxicity using the hen´s egg test chorio allantois membrane (HET-CAM) model.

L-ornithine decreases (= 1 mmol/L) the *de novo* urea synthesis of keratinocytes and increases (= 10 mmol/L) the expression of the urea-generating enzyme arginase. Otherwise there is no influence on the expression of ornithine decarboxylase, the initial enzyme of polyamine synthesis.

Furthermore there is no cytotoxic potential of L?ornitine up to 20 mmol/L. Higher concentrations induce apoptosis in native keratinocytes in a concentration- and time-dependend manner. *In vivo* L-ornithine HCl causes no vascular and no systemic reaction up to 100 mmol/L detectable by laser Doppler fluxmetry in the HET-CAM model. The dermal availability of L-ornithine after topical application of semi-solid standard-preparations containing 10 % L-ornithine HCl was investigated by using the FRANZ modell. Best results in the concentration-time-profil were received by the amphiphile system (Basiscreme DAC).

P113

L-carnitine promotes human hair growth in vivo and in vitro

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The zwitterionic alcohol L-carnitine plays a key role in the intramitochondrial transport of fatty acids for ßoxidation thus serving important functions in energy metabolism. We hypothezised that L-carnitine, which is frequently employed as a dietary supplement e.g. in sports, may also stimulate hair growth by increasing energy supply. Here, we show for the first time that carnitine-tartrate does indeed promote the growth of human hair scalp follicles (HF) in vitro and in vivo.HF in the anagen VI stage of the hair cycle were cultured in the presence of carnitine-tartrate (0,5-50ng/ml) for nine days. Hair shafts elongation was masured every other day and HFs were harvested for cryosectioning. At day 9, HFs treated 5ng/ml or 0,5ng/ml carnitin-tartrate showed a hair shaft elongation of 37% compared to only 25% in the control group. Significantly more HFs in the carnitine-tartrate treated group were still in anagen compared to controls. This corresponded to a downregulation of TUNEL positive (i.e. apoptosis) and a slight upregulation of Ki-67 positive (i.e. proliferation) cells in the hair matrix of carnitine-tartrate treated HF compared to vehicle controls.In a clinical double-blind trial 60 patients with androgenetic alopecia were treated topically with carnitine-tartrate 2% solution for six months. Evaluation of hair growth using the TrichoScan technique showed a significant increase in the total number of terminal scalp hairs and in the anagen/telogen ratio in patients receiving topical carnitine-tartrate. These data support our hypothesis, that L-carnitine is able to stimulate human hair growth by upregulation of keratinocyte proliferation and suggests the use of L-carnitine as an adjuvant form of treatment in the management of androgenetic alopecia and other forms of hair loss.

P114

***Borrelia burgdorferi* induces specific cutaneous infiltrates of B-cell lymphocytic leukemia - Remission of cutaneous infiltrates by ceftriaxon**

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There is increasing evidence that Borrelia burgdorferi may not only induce cutaneous pseudolymphomas but also low-grade malignant primary cutaneous B-cell lymphomas (PCBCL). This observation is based upon the intralesional detection of Borrelia DNA and the response of these PCBL to borreliacidal antibiotics. In addition, Borrelia DNA has also been detected in cutaneous infiltrates of B-cell chronic lymphocytic leukemia (B-CLL), i.e. in secondary cutaneous lymphomas. Here we show that Borreliae can be isolated from specific cutaneous infiltrate of B-CLL and that these infiltrates respond to borreliacidal antibiotic therapy.
A 57-year-old woman who had been suffering from BB-CLL for 7 years (RAI stage (cs) II) developed erythema migrans 2.5 years later. As the patient refused antibiotic treatment, acrodermatitis chronica atrophicans occurred later on her left hand and both legs. In addition two of her fingers swelled up, being infiltrated with tumor. Histologic examination of the skin of the involved fingers showed a dense monomorphic infiltrate consisting of small lymphocytes with hyperchromatic nuclei. PCR analysis of the immunoglobulin heavy chain gene was consistent with a monoclonal B-cell infiltrate. The clone identified in the skin was identical to that identified in the bone marrow of the patient. Spirochetes were successfully cultivated from this specific cutaneous infiltrate of B-CLL and genotyped as Borrelia afzelii. Treatment with ceftriaxon lead to a complete remission of all skin lesions except the cutaneous atrophy. Three years later the patient died from B-CLL without recurrence of any skin changes.
Our findings allow the following conclusions: (i) B-CLL lymphocytes possess residual immunological functions and recognize Borrelia antigens, (ii) Borrelia antigens can induce a specific cutaneous B-CLL infiltration and (iii) this infiltration responds to borreliacidal treatment. As Borreliae can induce both primary and secondary B-cell lymphomas of the skin, Borrelia infection should be considered in both of these diseases.

P115

Elevated serum levels of solube FAS ligand in patients with various cutaneous drug reactions

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Cutaneous manifestations belong to the most frequently observed adverse drug reactions. Clinical appearances include both relatively benign forms such as maculopapular rash (MPR) and potentially life threatening diseases such as Stevens-Johnson Syndrome (SJS) and toxic epidermal necrolysis (TEN). While absent in regular skin, in these diseases apoptosis of keratinocytes is a common histopathological feature. In TEN this process is thought to occur through an interaction between a cell-surface death receptor FAS (CD95) and its respective ligand (FASL). Furthermore, in TEN this interaction is reflected by elevated concentrations of soluble FASL (sFASL). In our study sFASL levels were determined by enzyme-linked-immunosorbent assays (ELISA) in patients with drug induced skin eruptions: MPR=46; erythema multiforme (EEM)=7; SJS=1 and TEN=2. As controls healthy volunteers (142) and patients with frequent skin diseases routinely seen and treated at our department such as erysipelas, atopic dermatitis, psoriasis, herpes zoster, acute exanthematous viral infections and urticaria (135) were analized. Elevated levels of sFASL were frequently detected in sera of patients with MPR (14), with EEM (2) with SJS (1) and with TEN (1) while it was below the detection limit in all sera of healthy controls and in almost all sera of the comparison group. In conclusion, sFASL could serve as a serologic parameter indicative of drug induced skin eruptions including MPR, EEM, SJS and TEN.

P116

Selective expression of chemokine monokine induced by interferon-? (MIG) in alopecia areata

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Alopecia areata (AA) is being characterized as a tissue-restricted autoimmune disease. Locally infiltrating lymphocytes have been implicated as effector cells being responsible for the reversible alteration of the hair follicle; however, the expression pattern of chemokines guiding leukocytes to sites of evolving AA has not been studied in detail yet.We here analyzed biopsies from 11 defined AA patients for the expression of chemokines presumably leading to recruitment of mononuclear cells such as IP-10, MCP-1, MIG, MIP-1a and ß and GROa by in situ-hybridization. We detected a strong expression of MIG mRNA, a moderate expression of MCP-1, and a weak expression of IP-10 mRNA whereas GROa, MIP-1a and MIP-1ß were not expressed at significant levels. MIG mRNA was mainly found in mononuclear cells in the peri- and intrabulbar infiltrate as well as in the follicular papilla; in these sections MIG mRNA expression spatially strongly correlated with infiltration of CD3+, CD4+ and CD8+ lymphocytes. In contrast to MIG, MCP-1 mRNA showed a completely different distribution with expression by keratinocytes of the inner root sheath but only weak expression around the hair follicles. Chemokine expression patterns did not appear to correlate with clinical severity and criteria defined by the National Alopecia Areata Foundation such as extent of hair loss, number of AA episodes or presence of associated diseases. Regarding leukocyte infiltration in AA, the following scenario might be feasible: Due to as yet unknown events autoreactive T helper lymphocytes interacting with their putative autoantigen in the anagen hair follicle induce MCP-1 expression by keratinocytes which then supports recruitment of additional T lymphocytes and monoyctes/macrophages to the immediate vicinity thus focussing the inflammatory reaction around the hair bulb. In turn, the latter are furthermore stimulated by interferon-?-producing T cells to synthesize MIG and, to a lesser extent, IP-10 which both perpetuate recruitment of lymphocytes thus promoting an ongoing inflammatory reaction.

P117

Development of new vehicle systems for cutaneous gene therapy

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Keratinocytes can be *in vitro* transfected by naked plasmid DNA. DNA applicated this way will be taken up by endocytosis and is followed by nonpermanent expression. This allows for a vital control of the therapeutic effect and offers a high degree of safety. Additionally, in avoiding viral vectors no immune reaction can be detected.
New nanoemulsions have a benefit compared to liposome systems. They enable for example a better penetration of highmolecular substances and even make the permeation of the Stratum corneum, caused by intensive interactions with the lipids of this barrier, possible. Hence higher transfection rates can be expected.
Such nanoemulsions have been created and their stability has been tested. These systems have also been characterized relating to their *in vivo* toxicity. Later evaluation of these formulations requires the development of a model for transfection of human keratinocytes.

P118

Familial mastocytosis associated with gastrointestinal stromal tumors: identification of a novel germline c-KIT mutation in exon 8 responding to imatinib in vitro

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Mastocytosis (OMIM 154800) represents a heterogenous group of diseases characterized by accumulation of mast cells in tissues. Sporadic mastocytosis in adults is usually associated with somatic mutations in exon 17 of c-KIT, affecting the enzymatic site and causing constitutive activation of the KIT tyrosine kinase. In contrast, the cause of familial mastocytosis is unknown. Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors usually caused by somatic c-KIT mutations that activate KIT by affecting regulatory portions of the molecule, leaving the enzymatic site unchanged. Most GISTs respond to KIT inhibitors that bind to the enzymatic site, unlike most mastocytosis cases whose modified enzymatic site is not affected by these drugs. In the present study, three members of a kindred with familial mastocytosis and GISTs were investigated. We identified a novel germline c-KIT mutation in exon 8, resulting in deletion of codon 419 and affecting the extracellular portion of KIT. In vitro studies showed that this mutation activates KIT and that the mutant KIT is inhibited by imatinib, a widely used drug that competitively inhibits wild type KIT and KIT activated by regulatory type mutations. Our findings demonstrate that not only sporadic, but also familial mastocytosis may be linked to activating c-KIT mutations. They identify a new regulatory region in the KIT molecule and suggest that patients with mastocytosis caused by regulatory type c-KIT mutations may respond to wild type KIT inhibitors.

P119

The influence of TH1-cytokines of the y^+-system expression in microvascular endothelial cells (HDMEC) and native keratinocytes (NHEK)

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As it is generally known that the distribution of TNF-alpha by keratinocytes, macrophages and other effector cells is increased in psoriatic lesion. Thus complex aftereffect can be derived the pathological overproduction of TNF-alpha. Latest findings related to the TNF-receptor associated signal transduction deliver more possibilities for antipsoriatic therapy. In different examinations we have shown that the hCAT2b, as one gene of the cationic amino acid transporter y^+-system, differ in its expression level by incubation of three TH1-cytokines (TNFa, INF? and IL-1ß). It is also known that hCAT2b is regulated by NF-?b and therefore can be stimulated directly in its expression by TNF-alpha. These relations have been examinated by real time PCR and immunhistochemically treatment of psoriatic skin for HDMEC and NHEK. Above all, it was possible to show that hCAT2a and hCAT2b experience a strong modification in their expression by incubation of the TH1-cytokine. If these relation supports a calculated therapeutic influence control of the inflammatory processes of psoriasis, further examinations have to show this.

P120

Activity of disease as an important new risk factor for naproxen-induced pseudoporphyria in juvenile idiopathic arthritis

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Pseudoporphyria (PP) is characterized by skin fragility, blistering and scarring in sun-exposed areas, mimicking the photosensitivity reactions seen in the cutaneous porphyrias but with normal porphyrin metabolism. The phenylpropionic acid derivative group of nonsteroidal anti-inflammatory drugs (NSAIDs) is known to cause PP. Naproxen, a member of this group, is the most commonly prescribed drug in the therapy of juvenile idiopathic arthritis (JIA). The incidence of PP, which we observed in a 10 years-period, was 11.4% in 395 patients treated with naproxen. A prospective cohort of total 196 patients (pts) (127 girls and 69 boys, ages 0-16) with JRA were studied comparing 3 groups PP+ (45 pts treated with naproxen developed PP), PP- (96 pts treated with naproxen had no PP), and a control group KO (55 pts not treated with naproxen).
In PP+, 65% of pts were diagnosed with the pauciarticular subtype of JIA (EOAP-JIA). This prevalence was significantly higher than in PP- (23%) and KO (4%). The mean age of onset of JIA in PP+ (4.5 y) was significantly lower compared with PP- (8.5 y) and KO (8.2 y). Increased risk factors for the development of PP were the mean dose of naproxen (>15mg/kg/day), an increase of the dosage and co-medications, especially chloroquine. Significant differences in laboratory tests between PP+ and PP- were lower hemoglobin (<11.75 g/dl), higher counts of leukocytes (>10,400/µl) and platelets (>408,000/µl), elevated ESR (>26mm/h), and elevated protein levels of urine. Skin phototype (I-II) and exposure to sunlight were confirmed to be risk factors for the development of PP. There were no significant differences between the groups in sex, eye colour, ANA positivity and other laboratory parameters.
Conclusion: JIA disease activity, as measured by haemoglobin, leukocytes, platelets and ESR, is an important risk factor in the development of PP. Especially in young children diagnosed with EOPA-JIA and high inflammatory parameters treatment with naproxen should be well considered and, if necessary, dosage should be limited to =15mg/kg/day. All naproxen-treated patients should be advised to sun-protection and regular thorough application of sunscreen.

P121

Comparison of T-cell receptor ß- versus ?-chain gene rearrangement analysis in the differentiation of parapsoriasis and early-stage mycosis fungoides

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Clonal T-cell receptor (TCR) rearrangement by PCR-based GeneScan analysis (GSA) was shown in previous studies to be a valuable diagnostic technique with an overall clinical sensitivity of 76% (TCR-?) to 98% (TCR-ß) in patients with advanced CTCL. Clinical sensitivity for TCR-? GSA was 19% in parapsoriasis and 67% in early-stage mycosis fungoides (MF) patients. The aim of this study was to determine the value of TCR-? GSA in comparison with TCR-? GSA in differentiating between parapsoriasis and early-stage MF (clinical stages Ia and Ib). Formalin-fixed and paraffin-embedded skin biopsies of 12 patients with clinically and histologically diagnosed parapsoriasis (2 woman and 10 men, aged 60-80 years, mean 70) and 11 patients with early-stage MF (3 woman and 8 men, aged 15-96 years, mean 62) were investigated for TCR-? and -ß chain gene rearrangement. Furthermore, the patients were examined for blood involvement. Tissue specimens were analysed using a semi-nested TCR? (ß) PCR with consensus primers covering the V?1 to V?8 (Vß1 to Vß65) segments and the primers JGT1/2 (JßFS1A/JßFS1) and JGT3 (JßFS2A/JßFS2) for the J region. Detection and size determination of PCR products was done by high-resolution fragment analysis (GSA). To avoid pseudomonoclonality in interpretation of the results each sample was analysed twice with each primer pair. Monoclonality was found in 25% (3/12, TCR-?) vs. 63% (7/11, TCR-ß) in the skin of parapsoriasis and in 55% (6/11, TCR-?) vs. 80% (8/10, TCR-ß) in MF patients. The detection rate of monoclonality in the peripheral blood was 17% (2/12, TCR-?) vs. 8% (1/12, TCR-ß) in parapsoriasis and 27% (3/11, TCR-?) vs. 40% (4/10, TCR-ß) in MF patients. However, none of the identified clones in the peripheral blood was identical with a clone detected in the corresponding skin sample. The clinical sensitivity of TCR-ß GSA is increased in parapsoriasis and early-stage MF in comparison with TCR-? GSA. However, the increased sensitivity is associated with a decreased specificity. Thus, application of TCR-ß GSA offers no additional help to clearly dissect borderline cases like parapsoriasis and early-stage MF.

P122

Cancer Regression induced by Immunostimulatory CpG Oligodeoxynucleotides in Patients with Metastatic Melanoma, the Results of a Clinical Phase II Trial

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Stimulation of Toll-like receptor (TLR) 9 by pathogen-derived compounds leads to direct activation of human antigen-presenting plasmacytoid dendritic cells (pDC) and B cells and indirectly dramatically increases cytotoxic T and natural killer cell responses. The synthetic oligodeoxynucleotide CPG 7909 contains CpG motifs optimized to specifically interact with TLR9 and is a strong activator of both innate and specific immunity. CPG 7909 crossreacts with mouse TLR9 and has shown impressive antitumor activity in preclinical tumor models when used as monotherapy.
In a clinical phase II trial in stage IV melanoma patients (n=20), we applied a fixed dose of 6 mg CPG 7909 s.c. at weekly intervals. To particularly trigger tumor-specific adoptive immunity, we preferentially injected CPG 7909 in the surrounding of peripheral lymph nodes draining tumor-bearing skin areas. This treatment induced objective tumor responses -as assessed by EORTC-RECIST guidelines- in 2/20 patients and significant antitumor activity in further 3 patients. Adverse events included transient injection site reactions, fever, and arthralgias. Hematological and non-hematological toxicities were limited. So far (7/20pts.), FACS-based phenotyping of PBMC revealed consistent activation of BDCA-2+ pDC with increased CD86 and HLA-DR expression, activation of CD4+ T cells with increased numbers of CD4+CD40L+ cells, and an increased frequency of CD19+CD38++ plasma cells (IgM-producing) during CPG 7909 therapy. In the one responding patient analyzed thus far, additional changes with increased numbers of activated CD8+CD40L+ T cells as well as CD8+CCR7-CD45RA- effector memory cells and decreased frequencies of IL-10-producing CD4+ and CD8+ T cells could be observed. Consistent changes in NK cell activity were not detected, assays to determine frequencies of antigen-specific T cells are ongoing. We conclude that CPG 7909 monotherapy exerts objective anti-tumor activity in melanoma patients, is well-tolerated, can be applied safely and induces a phenotypic signature in PBMC associated with exposure and, possibly, response to therapy.

P123

Systemic rituximab therapy in the treatment of primary cutaneous B-cell lymphomas

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According to the EORTC classification for primary cutaneous lymphomas primary cutaneous B cell lymphomas (PCBCL) are classified among the non-Hodgkin-lymphomas as indolent PCBCL (marginal zone lymphomas and follicle center cell lymphomas) and as intermediate lymphomas (large B cell lymphoma) (Willemze R,Blood, 1997). Because PCBCL are relatively rare, no standard therapy exists which could be evaluated based on evidence in a larger number of patients. So far excision and local radiotherapy is widely accepted as first line treatment in primary cutaneous B cell lymphomas.
This report comprises 11 male CBCL patients treated with intraveneous rituximab including a long observation period of 36 months. 10 patients had an indolent lymphoma including 9 patients with a follicle center cell lymphoma, one patient with the history of a marginal zone lymphoma. A further patient suffered from an immunocytoma.

Only adult patients with the history of a histological confirmed and relapsed PCBCL with extensive tumor burden (area > 10cm^2 or multiple lesions) were enrolled on this applicational observation study. Also a secondary systemic involvement by the B cell lymphoma was allowed. All patients never had received rituximab before. The antibody was supplied in vials as 100mg/10ml rituximab. In the pharmacy of the Charité hospital a solution containing a concentration of 1mg/ml was prepared. A dosage of 375mg per m^2 body surface per cycle was intended. The treatment was scheduled for the duration of 8 cycles.
The median duration of clinical remission is up to now, with 4 progression free patients still being under observation. The follow up interval of two of the four patients, who are still under observation, is 7 and 20 months, respectively, and of the remaining two patients already 30 months. The duration of clinical remission in the other five patients lasted 4, 8, 11, 23, 24 months. Two patients had a progressive disease under rituximab treatment.
In conclusion, it could be shown in this subset of patients with relapsed cutaneous B cell lymphoma, that the treatment of primary cutaneous B cell lymphomas with rituximab is well tolerated, feasible, easily applicable, and in its prolonged regimen connected with prolonged remissions.

P124

Unperturbed wound healing in IL-15 deficient mice

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For the survival of an indiviuum an intact skin is essential. The skin protects the organism against water loss and the invasion of viruses and bacteria. Wound repair is a tightly regulated and complex process and involves different celltypes, cytokines and growth factors (Clark, 1996; Martin, 1997). Recently, a role of ?d dendritic epidermal T-cells (DETC) in wound healing has been demonstrated and it has been suggested, that ?d DETC are important to ensure a correct growth factor environment, in particular to mediate keratinocyte proliferation through KGF (Jameson et al., 2002). IL-15 is a survival factor for ?d-DETC. The IL-15 receptor (IL-15 R) consist of three subunits, the IL-15 a-chain, IL-2ß-chain and the IL-2 ?c-common chain (Giri et al., 1995a; Giri et al., 1995b). Deletions in the IL-15, IL-15 Ra or IL-2ß locus lead to an impaired development of ?d-T cells in epithelia (Ye et al., 2001; Suzuki et al., 1997; Lodolce et al., 1998). It was shown that transplantet ?d DETC were not able to survive in the skin of IL-15 knock out mice (De Creus et al., 2002). In addition, mice lacking ?d DETC in the skin showed a delayed wound repair (Jameson et al., 2002). We investigated the effect of an IL-15 deficiency in mice on the repair of superficial and full thickness skin wounds. Superficial wounds were induced by tape-stripping and the repair rate was determined by the recovery in transepidermal water loss. The repair rate in full thickness wounds 10 mm in diameter on the flank of the mice was determined by measurements of the wound size using a computerized digital imaging system. In addition, wound contraction and reepithelization was evaluated. The repair rate in superficial wounds of IL-15 deficient mice did not show significant differences compared to wild type mice. Also, the healing rate in full thickness wounds, neither overall wound size nor wound contraction or reepithelization were different in deficient compared to wild type mice. We suggest that the lack of ?d DETC may be compensated by other mechanisms. In summary, IL-15 deficiency does not effect superficial or full thickness wound healing in our model.

P125

The Interleukin-6 Cytokine System Regulates Epidermal Wound Healing

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`Interleukin-6 (IL-6) is involved in the growth and differentiation of numerous cell types. In the skin it is produced primarily by keratinocytes. The transcription factor STAT3 is activated by cytokines of the IL-6 family. In the present study we examined the involvement of IL-6, soluble IL-6-receptor, and STAT3 in epidermal wound healing after injury by tape-stripping. After injury in wild type mice we found an increased immunostaining of IL-6 and IL-6R on epidermal keratinocytes at 15 min to 5 h after treatment. The increase in IL-6 and IL-6R was confirmed by Western blotting using epidermal homogenates. In IL-6 deficient mice, epidermal wound repair was reduced at 3 to 24 h after treatment. Topical application of IL-6 or Hyper-IL-6, a complex of IL-6 linked to the soluble IL-6 receptor, enhanced the repair process in wild type mice. Application of the fusion protein gp130-FC, a specific inhibitor of the agonist IL-6/sIL-6 receptor complex, delayed the repair in wild type, but not in IL-6 deficient mice. STAT3 tyrosine phosphorylation was induced after injury in wild type, but markedly reduced in IL-6 deficient mice. Our results indicate that the IL-6 cytokine system, particularly transsignalling via the soluble IL-6R, is critically involved in epidermal wound repair.`

P126

The intracellular calcium concentration and the differentiation of HaCaT-cells is influenced by N-methyl-D-aspartate-receptors

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Ionotropic glutamate receptors (ligand-gated ion-channel proteins) of the N-methyl-D-aspartate-receptor type could enable a transmembraneous calcium influx from the extracellular space. The present study was performed to investigate the possible influence of NMDA-receptors on the intracellular calcium concentration, proliferation and differentiation of HaCaT-cells.

The intracellular calcium concentration of HaCaT-cells was studied under the influence of the selective agonist N-methyl-D-aspartate (NMDA) and the selective NMDA-antagonist MK-801. Measurement of the intracellular calcium concentrations was performed by means of laser scanning microscopy. The proliferation was investigated using the crystal-violet-method. The expression of cytokeratin 10 after blocking NMDA-receptors with MK-801 was used for differentiation studies.

Using NMDA, there was a significant increase in the number of cells showing elevated intracellular calcium concentration at a dose between 25µM and 1mM (up to 84,6%). The NMDA-associated calcium influx could be significantly suppressed by prior application of 100µM MK-801. There was no influence of NMDA-receptors on the proliferation of HaCaT-cells. There was also no cytotoxic effect of NMDA. The expression of cytokeratin 10 could be suppressed by blocking NMDA-receptors with MK-801.

The examinations show that glutamate receptors of the NMDA-type influence the differentiation of HaCaT-cells by regulating the keratinocytic calcium concentration.

P127

Treatment of recalcitrant pustular psoriasis with infliximab: effective suppression of selective chemokines

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Tumor necrosis factor-a (TNF-a) plays a major role in the pathophysiology of psoriasis. Good clinical responses of psoriasis to anti-TNF-a-based therapies have recently been demonstrated. We here for the first time provide an in situ-analysis of chemokine modulation by the TNF-a-antagonist infliximab. A 61-year old male patient with a 2-year history of recalcitrant pustular psoriasis of von Zumbusch type who did not respond to conventional therapies rapidly resolved upon treatment with infliximab. Using in situ-hybridization, we demonstrate that mRNA levels of chemoattractants interleukin 8 (IL-8) and growth-related oncogene-a (Gro-a) which are responsible for the recruitment of neutrophils to sites of evolving pustules are rapidly down-regulated upon blocking TNF-a. The expression of monocyte chemoattractant protein-1 (MCP-1) was suppressed as well. In contrast to observations made in plaque psoriasis, mRNA expression of monokine induced by interferon-? (Mig) or interferon-inducible protein-10 (IP-10) could not be detected in pustular psoriasis. We conclude that anti-TNF-a-treatment with infliximab is an effective therapy in severe pustular psoriasis which presumably acts by down-regulation of disease-promoting chemokines such as IL-8, Gro-a and MCP-1. Chemokines IP-10 and Mig which are expressed under situations where a Th1 immune response is prevailing could not be detected suggesting that TNF-a-dependent mechanisms apart from or additionally to Th1 immune reactions are operative in pustular psoriasis.

P128

Influence of NSAIDs on oxidized eicosanoids as analysed by GC-MS in microdialysates of UVB-irradiated human skin and in cultured keratinocytes

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UVB-irradiation of human skin leads to the induction of a cascade of inflammatory reactions including the release of oxidized arachidonic acid mediators. These can be formed either non-enzymatically via free radical attack and lipid peroxidation of phospholipid-bound arachidonic acid or enzymatically via inflammation-induced liberation of arachidonic acid from membrane phospholipids and subsequent formation of prostaglandins and leucotrienes. The relevant enzymes for this conversion are cyclooxygenase isoenzymes and 5-lipoxygenase, which can be inhibited by anti-inflammatory drugs like diclofenac, acetylsalicylic acid and COX-2 inhibitors.

We studied the UVB-induced release of eicosanoid lipid mediators and the influence of diclofenac on this release *in vitro* in cultured keratinocytes (HaCaTs) and *in vivo* in dermal interstitial fluid obtained by cutaneous microdialysis technique. Therefore the volar forearm of healthy volunteers was exposed to UVB-irradiation. Afterwards diclofenac was applied topically under occlusion. After 3 h or 24 h microdialysis cathethers were inserted intradermally and perfused with a isotonic electrolyte solution. The collected samples were analysed for oxidized eicosanoids (8-iso-PGF_{2a}, PGF_{2a}, PGE_2, monohydroxyeicosatetraenoic acids as HETEs and LTB_4) using GC-MS. These could be detected and quantified in the microdialysis samples with enhanced levels of isoprostanes and PGE_2 24 h after UVB-irradiation and of HETEs and LTB_4 after 3 h. Topically applied diclofenac reduced these levels to different extents. In keratinocytes a UVB-dose dependent release of oxidized eicosanoids could be demonstrated 24 h after UVB-irradiation, which was reduced by application of diclofenac.

Keratinocytes have been shown to be a suitable *in vitro* model to investigate the effects of UVB-irradiation and NSAIDs on the release of lipid mediators in cell cultures. Cutaneous microdialysis is a minimally invasive technique allowing to study endogenous eicosanoid derivatives in the interstitial spaces of the dermis in a variety of human inflammatory skin disorders.

P129

Cytokine modulation in adjuvant IFN-alpha therapy of melanoma

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IFN-a therapy is well established in adjuvant therapy of melanoma. Its mode of action comprises inhibition of tumor cell proliferation and enhancement of phagocytosis and specific cytotoxicity. Cytokine induction or modulation by IFN-a might by itself be part of the therapeutic efficacy and might be responsible for the plethora of side effects. In order to get more insight into IFN-a modulated cytokines we examined the cytokine profiles in patients undergoing adjuvant IFN-a2b therapy for stage II or III melanoma and who received either 10 Mio I.U. or 5 Mio I.U. three times a week for up to two years. Serum was examined before treatment, after 4 weeks and thereafter every 3 months. Time course analysis of IL-1ß, IL-2, hsIL-2R, IL-6, IL-10, TNF-ß, beta-2 microglobulin (b2M) could be obtained in 35 patients by using specific ELISAs. The median values for the whole study group were 4,8 pg/ml (range 0-2) for IL-2, 5,1 pg/ml (range 3,0-8,9) for IL-6, 3,38 pg/ml (range 0,37-23,6) for IL-10, 11,5 pg/ml (range 0-196,5) for TNF-a and 1,68 mg/L (range 0,86-3,37) for b2M. IL-1ß could neither be detected before nor under therapy in the majority of patients. TNF-a showed a significant increase after 4 weeks of therapy ($p<0,05$). In patients who developed metastases significantly lower TNF-a levels were detected both pre-treatment as well as while uncder treatment. Highly significant elevated serum values for TNF-a were found in patients in whom IFN-a2b therapy had to be discontinued because of severe side effects. In the majority of patients significant increases were detected for b2M which can serve as a biological marker for IFN-activity. A decrease of serum IL-2 was found while hsIL-2R was elevated. IL-10 showed increased values after 3 months of therapy with patients developing progressive disease exhibiting the highest increases. Our results indicate that even after prolonged IFN-a therapy significant modulations in cytokine levels can be found. Low TNF-a values both pre- as well as under treatment and increased IL-10 level under therapy are indicative for high risk of relapse while b2M levels can serve as a marker for IFN-a therapy.

P130

Down-regulation of VIP receptor expression in atopic dermatitis

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G-protein-coupled receptors are a major pathway for signal transduction between cells but have not been associated with the pathogenesis of human immune disease so far. As receptors for vasoactive intestinal polypeptide (VIP) have recently been suggested to play a key role in immuno-modulation, we studied the expression of the $VPAC_2$ in the human immune disease atopic dermatitis (AD). In situ hybridisation and immunohistochemistry demonstrated $VPAC_2$ mRNA and protein expression in human mast cells surrounded by VIP positive nerve fibers. Gene array experiments and RT-PCR studies revealed high levels of $VPAC_2$ mRNA expression in mast cells which were increased compared to other receptors such as VPAC1 or VIP in the human mast cell line HMC-1. Stimulation of HMC-1 cells lead to a downregulation of $VPAC_2$. Similarly, quantitative immunohistochemistry for $VPAC_2$ in atopic dermatitis sections revealed a significantly decreased $VPAC_2$ immunoreactivity in mast cells. The downregulation of $VPAC_2$ mRNA and protein in human mast cells in atopic dermatitis suggests a pathogenetic role of a G-protein-coupled receptor in human immune disease.

P131

Expression Profile of Different Antimicrobial Peptides in Psoriatic Skin

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Psoriasis is a chronic inflammatory skin disease where, despite the high amount of bacteria colonizing the lesions, skin infections are rare. Therefore we addressed the question which phenomenon could be responsible for this observation. Recently, a number of antimicrobial peptides (AP) were identified from human skin. Some of these peptides are known to be constitutively expressed whereas others are induced after stimulation with proinflammatory cytokines or after contact with microorganisms. The current study was initiated to investigate the mRNA expression of different AP in patients with psoriasis vulgaris.

Skin samples were obtained from untreated patients with chronic plaque-type psoriasis (n=15). Two punch biopsies were taken from each patient, one from the margin of a lesion, the other one from non-involved skin. Normal human skin from patients undergoing surgery of benign skin tumours served as controls (n=18).

An optimised protocol for sample storage and RNA extraction was established. Realtime-RT-PCR experiments were performed with intron-spanning primers for the following AP: human b-defensin (hBD)-2 and -3, RNase 7 and Psoriasin.

A higher transcription level for hBD-2-, -3-, RNase 7- and Psoriasin-mRNA was identified in all lesional skin samples as compared to uninvolved skin of psoriasis patients. Interestingly, all molecules under investigation were also shown to be expressed at a higher transcription level in uninvolved skin as compared to the skin of healthy people.

In conclusion we found that HBD-2-, -3-, RNase 7- and Psoriasin-mRNA is significantly induced *in vivo* in psoriatic skin. The expression of these antimicrobial peptides may contribute to the low rate of skin infections observed in patients with psoriasis.

P132

Polymorphism of Candida albicans : a major factor in the interaction with human dendritic cells

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The fungus Candida albicans, a polymorphic fungal organism behaves as a commensal as well as a true pathogen of areas highly enriched in dendritic cells (DCs). Morphological plasticity is a common feature of many pathogenic fungi and has long been considered to be a major determinant of virulence in C. albicans. One of the most extensively studied in vitro models for polymorphism is the temperature and pH-dependent transition from yeast to (pseudo-)hyphae in C. albicans. Morphological plasticity of C. albicans has been shown to influence the immune reaction triggered within the human host. A protective immune reaction in candidiasis requires the induction of a Th1 type response whereas a Th2-response is associated with persistence of the infection. DCs play a major role in the regulation of an immune-response towards either direction and are likely to have a major impact on the outcome of fungal infection. Here we show, that human monocyte derived DCs discriminate between yeast and hyphae. Whereas yeast cells are readily phagocytosed and at least partially degraded within human DCs, hyphae of the same strains are capable to evade DCs. Intracellular filamentation of phagocytosed yeast cells results in complete destruction of the DCs 12h post infection. Both morphotypes differ significantly with regard to the pattern of cytokine secretion induced by DCs. IL-8 and TNF-alpha are induced to a much higher degree by hyphae than by yeast forms. However, whereas this seems to depend on purely the cell mass in the case of IL-8, TNF-alpha induction is strongly dependent on the morphotype.

P133

In-vitro susceptibility of Malassezia furfur and Malassezia pachydermatis against Fluconazole and Ketoconazole by means of the E-test®

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Objectives: Malassezia furfur and Malassezia pachydermatis are anthropophilic fungi with complex growth requirements. Apart from their physiological appearance on human skin they are causative agents of several skin disorders. Malassezia furfur occurs in both hyphal and yeast forms in skin lesions and in culture, but it has been observed only in yeast phase in normal human skin and in tissues from systemic infections. Malassezia pachydermatis exists only in yeast form, and a hyphal stage of this species has not been described.
Methods: The reference strain of Malassezia furfur (DSM 6170) and Malassezia pachydermatis (DSM 6172) were investigated. The minimum inhibitory concentrations (MIC) for fluconazole and ketoconazole were determined using the E test. The E test based on a combination of dilution and diffusion test in which plastic strips were impregnated with increasing concentrations of antifungal agents.
Results: The most effective agents to inhibit growth of Malassezia furfur and Malassezia pachydermatis in vitro were ketoconazole (median 0,013µg/ml). Also for fluconazole (0,39µg/ml) a low MIC value was found.
Conclusions: The data indicate the high in vitro activity of azole antifungals against Malassezia furfur and Malassezia pachydermatis.

P134

Expression Profiling Reveals Sets Of Genes Regulated Differentially In Hacat Keratinocytes Following Exposure To Secreted *Pseudomonas* Products

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We have shown previously, that the skin is able to defend infection by constitutive and upregulated production of antimicrobial peptides and that the extent of microbial induced keratinocyte defense gene expression also depends on microbial strains and growth conditions. For a better understanding of the mechanisms in the hostile interaction between keratinocytes and *Pseudomonas aeruginosa* and to determine the parameters for successful protection against infection we compared alterations of (HaCaT) keratinocyte gene expression upon exposure to supernatants of *P. aeruginosa* differing in their ability to induce expression of the antimicrobial peptide hBD2.

Differentially expressed keratinocyte genes were identified using an inflammation specific DNA array. The results were validated using real-time (kinetic) reverse transcription-polymerase chain reaction (RT-PCR). From 376 genes present on the inflammation specific DNA-array 36 were identified to differ in keratinocyte expression by a factor of at least 2 following stimulation. The identified genes could be grouped according to their expression pattern in genes upregulated following stimulation with the hBD2-inducing *P. aeruginosa* supernatant ("Set 1": e.g. Psoriasin, Calgranulin), genes upregulated following stimulation with a mixture of hBD2 inducing and hBD2 not inducing *P. aeruginosa* supernatant ("Set 2": e.g. MCP1, IL-8) and in genes down regulated following stimulation with this *P. aeruginosa* supernatant mixture ("Set 3": Integrin-ß4, HSPB1).

The absence of upregulation of those genes induced by hBD2 inducing *P. aeruginosa* supernatant in the presence of *P. aeruginosa* supernatant not inducing hBD2 gene expression indicates the presence of multiple *P. aeruginosa*-derived factors which a) are differentially secreted depending on *P. aeruginosa* growth conditions and b) differentially affect keratinocyte defense mechanisms. Future experiments will focus on the identification of those *P. aeruginosa* factors specifically affecting keratinocyte innate defense gene expression and on the identification of those keratinocyte genes specifically affected in their expression by these factors.

P135

Surveillance of MRSA-colonization in dermatology outpatients with inflammatory and non-inflamatory skin diseases

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During the past twelve years there was an increase of prevalence for methicillin-resistant *Staphylococcus aureus* (MRSA) in German hospitals, but only few publications report MRSA in dermatology, mainly focussing on hospitalised patients. To investigate the prevalence of MRSA in a dermatology outpatient setting a prospective study was performed. Anterior nares, perineal region and lesional skin was sampled in 229 patients with inflammatory skin diseases (n=88), venous leg ulcers (n=58), and basal cell carcinoma (n=83). Swabs were processed using standard methods. The isolated MRSA strains were characterised by pulsed-field gel electrophoresis after digestion with the restriction enzyme *Sma*I. The most MRSA-positive patients were amongst those with inflammatory skin diseases (10/88). This included one patient out of 32 with atopic eczema. Eight patients out of 88 with inflammatory dermatoses were under 16, only 1/8 with a dermatitis of the vulva proved to be MRSA-positive. Seven out of eight suffered from atopic eczema and were MRSA-negative.
5/58 patients with venous leg ulcers were MRSA positive, but none of the individuals with basal cell carcinoma. Molecular typing of the 14 available MRSA isolates revealed six different PFGE profiles. Half of the characterised isolates were identified as the Rhine-Hessen-epidemic strain, which is observed in 50% of MRSA isolates of this region. One strain was identified as the northern German epidemic strain and was the only one which was multi-resistant. The remaining isolates were sporadic MRSA-strains with unique PFGE profiles. None of them was resistant to vancomycin, gentamicin or mupirocin. Resistance to sulfamethoxazole/trimethoprim and doxycycline was rare, whereas resistance to levofloxacin and erythromycin was common. Statistical analysis revealed that the presence of chronic ulcers and erosions was significantly associated with MRSA-positivity. MRSA is uncommon in outpatients in our Dermatology clinic. However, patients with chronic ulcers and erosions are at risk and should be screened for MRSA colonization to implement infection control measures.

P136

***Staphylococcus aureus* strongly induces mRNA of the antimicrobial protein RNase 7 and proinflammatory cytokines in the human keratinocyte cell line HaCaT**

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The gram-positive bacterium *Staphylococcus aureus* is a major cause for superinfections in atopic dermatitis. Whereas keratinocyte-derived corneocytes represent the physical barrier against invading bacteria, little is known about the role of keratinocytes as effector cells. Our previous studies showed that gram-negative bacteria such as *Pseudomonas aeruginosa* produce pathogen-associated molecules (PAMs) that induce antimicrobial proteins (like the beta-defensin hBD-2) and proinflammatory cytokines in human keratinocytes. Therefore, we asked if gram-positive bacteria also induce these ?defense molecules?. The aim of this study was to analyse the production of antimicrobial proteins and proinflammatory cytokines in keratinocytes in response to *S. aureus*-derived pathogen-associated molecules.Culture supernatants of *S. aureus* grown under different conditions where used to simulate the human keratinocyte cell line HaCaT. The mRNA expression in HaCaT cells was measured by Realtime-PCR (LightCycler). Supernatants of bacterial suspension cultures as well as of static cultures resulted in strong IL-8 induction. In contrast, only cultivation under static conditions yielded high induction of RNase 7 and TNF-alpha, indicating a different regulation of induction of IL-8 vs. RNase 7 and TNF-alpha. To determine the time point when the production of the inducing PAMs were maximal, we conducted a time kinetic. After 4 and 5 days of static culture we observed the highest induction of IL-8 and RNase7. The inducers of IL-8 and RNase 7 could be precipitated from the supernatant with 40 % acetonitrile. Ion exchange chromatography showed that the IL-8 inducing PAMs are anionic and size exclusion chromatography indicated a molecular mass > 75 kDa. We are currently further characterizing these PAMs to understand more about the cross talk between microorganisms and human keratinocytes.We conclude from these findings that - depending on culture conditions - *S. aureus* secretes PAMs that can be recognised by skin keratinocytes leading to a rapid innate epithelial defence reaction through the production of inducible antimicrobial proteins and proinflammatory cytokines.

P137

Analysis of the prevalence and RFLP subtyping of Borrelia burgdorferi sensu lato in Ixodes ricinus ticks from a Lyme borreliosis endemic area in Europe

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Objectives: (i) To determine the prevalence of Borrelia burgdorferi (Bb) in Ixodes ricinus ticks in an highly endemic area for Lyme borreliosis (LB) in Europe (city of Graz, Austria) by polymerase chain reaction. (ii) To identify the borrelia genotypes present in those ticks by restriction fragment length polymorphism (RFLP) analysis.
Methods: 131 ticks were collected in June 2002 by the flagging method in a woodland recreation area of Graz. All ticks were placed into humidified tubes and stored at 4°C until separate DNA extraction for each tick with a commercial preparation kit (Roche). Extracted DNA was analysed using a semi-nested polymerase chain reaction with 2 primer pairs specific for an 818 bp fragment of the Bb ospA gene to detect infection of ticks followed by RFLP analysis with 5 endonucleases for genotype identification of positive samples.
Results: 27/131 (21%) ticks were found to be positive for Bb, 14 of which were nymphs and 13 were adults. RFLP analysis revealed 18 (67%) ticks to be infected with the genotype B. afzelii, 5 (19%) with Bb s.s., 3 (11%) with B. garinii, and 1 (3%) with B. valaisiana.
Discussion: (i) A high percentage of ticks in Graz is infected with Bb, indicating a considerable risk to contract LB after a tick bite in this area. (ii) Tick nymphs have the potential to transmit Bb, which is of practical importance as they are hardly visible by the naked eye. (iii) B. afzelii, the main causative agent of dermatoborrelioses, is the most prevalent genotype in ticks, consistent with the fact that dermatoborrelioses are the most frequent manifestations of LB in Europe. (iv) Bb s.s. is found more frequently than expected in our area. (v) B. valaisiana, which may have the potential to cause erythema migrans, was detected for the first time in Austria in this study.

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Direct vaccination with TAT-LACK fusion protein protects against murine cutaneous leishmaniasis

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Leishmaniasis is one of the most important infectious diseases worldwide. However, an efficacious vaccine does not exist at present. Protective immunity depends on IFN?-producing $CD4^+$ Th1- and $CD8^+$ Tc1-cells. We utilized the protein transduction domain (PTD) of HIV-1 TAT-protein to translocate proteins into the cytosol of dendritic cells (DC), thus faciliating MHC class I-dependent antigen presentation. We generated a fusion protein comprised of PTD-TAT and the *Leishmania* antigen LACK. As reported recently, susceptible BALB/c as well as resistant C57BL/6 mice vaccinated with TAT-LACK-transduced DC showed protective immunity against cutaneous leishmaniasis. However, DC-based vaccinations are expensive, laborious and therefore hardly applicable in *Leishmania*-endemic countries. Thus, we now utilized TAT-LACK as a direct antigen-specific vaccine with the intention to target DC *in vivo*. BALB/c mice were immunized twice i.d. into the ear with TAT-LACK or appropriate controls. One week later, infections were initiated i.d. into the other ear using standard high dose (HD, $2x10^5$ metacyclic promastigotes/ ear) as well as physiologically more relevant low dose (LD, 1.000 metacyclic promastigotes/ ear) inocula. We observed that administration of CpG oligonucleotides as adjuvant was necessary to obtain protective immunity. 5 weeks after HD infection, lesions in TAT-LACK-treated BALB/c mice were 24% smaller than those in PBS-treated mice. Interestingly, treatment with LACK alone had no beneficial effect on disease development compared to irrelevant controls. More importantly, in LD infections, lesion volumes were decreased by 75% in TAT-LACK- and 36% in LACK-treated groups. Our results confirm that priming of *Leishmania*-specific $CD8^+$ T-cells, in addition to $CD4^+$ cells, may be beneficial for the development of protective immunity, especially in the setting of natural low dose transmissions initiated by the bite of the sandfly. Our data also demonstrate that TAT-LACK fusion proteins can target DC *in vivo*. Future studies will focus on identifying the molecular mechanisms as well as the optimal antigens to be used in this effective TAT-fusion protein-based vaccination strategy against cutaneous leishmaniasis.

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A role for IL-1 receptor type I (IL-1RI) signalling in T helper (Th) education and for protective immunity in cutaneous leishmaniasis

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We and others have reported recently, that proinflammatory IL-1a/ß - perhaps released by infected dendritic cells (DC) - plays a critical role (together with IL-12) in the induction of protective Th1 immunity against cutaneous leishmaniasis [JEM 198:191 (2003)]. *L. major*-infected DC of BALB/c mice produced significantly less IL-1a than C57BL/6 DC, and treatment with IL-1a during T-cell priming induced Th1-mediated protection of progressive disease in otherwise Th2-prone BALB/c mice. IL-1a administration also led to enhanced protection of C57BL/6 mice. Since the receptors mediating IL-1 effects in this system have not been characterized, we studied IL-1RI-deficient C57BL/6 mice. C57BL/6 IL-1RI-/- or wild type mice were treated with IL-1a (50 ng) during days 1-3 post infection and lesion development was followed over the following weeks. In IL-1RI-deficient mice, no effect of IL-1a treatment was found, whereas IL-1a-treated control C57BL/6 mice showed significantly enhanced Th1 immunity associated with reduced lesions at several time points. We next analysed the phenotype of IL-1RI-/- mice in cutaneous leishmaniasis. IL-1RI-/- or wild type controls were infected with standard high dose ($2x10^5$) or physiologically more relevant low dose inocula (10^3 metacyclic promastigotes of *L. major*) intradermally and lesions were monitored for 3 months. Lesion sizes in infected IL-1RI -/- mice were not different from those in controls at any time point in high dose infections. Surprisingly, with low dose inocula, IL-1R-/- mice showed significantly decreased lesion sizes compared to wild type controls reaching maximal volumes at wk 6 (12±2 vs. 22±2 mm^3, $p<0.05$, n=5) with corresponding decreased lesional parasite loads, increased release of IFN? and reduced IL-4 production by lymph node T-cells at wk 6. These data indicate that IL-1RI is mediating the effect of DC-derived IL-1a in cutaneous leishmaniasis. In addition, IL-1RI signalling also plays a role in Th education and maintenance *in vivo* using low dose inocula of *L. major* in resistant C57Bl/6 mice. Future studies will analyse the role of IL-1a/ß during Th education and maintenance in more detail.

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Dipeptidyl peptidase IV (DP IV/CD26) is present on human SZ95 sebocytes and involved in regulation of DNA synthesis, differentiation and production of IL1-RA *in vitro*

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The ectopeptidase dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) is present on a wide variety of mammalian cells. It can act as an adhesion molecule, modulates biologically active peptides by cleaving dipeptides from the N-terminus and regulates T-cell activation. Using specific inhibitors of DP IV, it has been shown that this enzyme is also involved in the regulation of DNA synthesis of keratinocytes and lymphocytes and modulates lymphocyte cytokine production.
By flow cytometry, RT-PCR and enzymatic assays we could show for the first time that DP IV is present in the SZ95 human sebocyte line (CD26 expression 98 ± 1%, enzymatic activity 37.3 ± 9.7 pkat/10^6 cells). The DP IV inhibitors Lys[$Z(NO_2)$]-thiazolidide and -pyrrolidide suppress DNA synthesis of SZ95 sebocytes in a rapid and dose-dependent manner (IC50 55 µM and 92 µM; IC25 2.5 µM and 5 µM after 24 h of incubation). The differentiation measured by nile red fluorescence intensity was enhanced after 48 h of incubation with 50 and 100 µM.
Furthermore Lys[$Z(NO_2)$]-thiazolidide significantly induced the production of the IL-1 receptor antagonist (IL-1 RA) determined in SZ95 supernatants by ELISA after 24 and 48 h of incubation with a maximum 2-fold increase after 48 h. IL-1a was also detectable in low amounts but was not significantly changed in the presence of the inhibitor.
These data provide evidence that DP IV is involved in the regulation of sebocyte proliferation, differentiation and cytokine production and might in the future act as a therapeutic target in diseases with sebaceous hyperproliferation.
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A new concept for sweat gland innervation: expression of acetylcholine receptors

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According to the classical concept, sweat glands are innervated by sympathetic adrenergic nerves that change to the transmitter acetylcholine at the free nerve endings that surround the sweat gland. To date, it has been unknown which acetylcholine receptors are expressed by sweat gland myoepithelial cells, acinar cells and sweat duct cells and are hence controlling sweat production and expulsion. It is well established that the antimuscarinergic compound atropine, either applied locally or systemically can block hyperhidrosis. On the other hand, nicotine, acetylcholine (Ach) and other cholinomimethic drugs can induce sweating when injected into human skin. We therefore examined the expression nicotinic and muscarinic acetylcholine receptors in human skin with own and commercially available antisera immunohistochemically.
In myoepithelial cells of eccrine sweat glands we found a prominent expression of alpha 3, alpha 7, beta 2, beta 4 nAch-R and m4 and m5 mAch-R while alpha 5, alpha 9, beta 1, m1 and m3 isoforms were only weakly expressed, if at all. In the acinar cells we could only demonstrate the presence of alpha 9, beta 2, m1 and m3 isoforms while the reactivity of the other Ach-R was very weak. The sweat duct shows strong expression of alpha 3, alpha 7, beta 2, beta 4, m1, m3, m4 and m5. Being rich in choline acetyltransferase, myopepithelial cells not only serve as receptors for Ach derived from sympathetic nerve endings but produce Ach themselves while apparently acinar cells produce only minute amounts of choline acetyltransferase and thus acetylcholine. The exact function of myoepithelial cells is unknown, but the classic concept is the expulsion of preformed sweat by contraction. We therefore conclude that sweat production and sweat release is under control of both, nicotinic and muscarinic Ach-R.

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Evidence for the biological relevance of UVA-induced, enzyme-independent nitric oxide formation in healthy human skin

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Nearly all dermal cells are capable for NO production either constitutively by the endothelial and neuronal NO-synthases or after cytokine-challenge as well as during inflammation by the inducible isoform of this enzyme family (iNOS). NO plays a pivotal role in human skin erythema and edema formation, wound healing, melanogenesis, cytotoxicity, cell proliferation and differentiation, among others. Under the specific conditions of low pH or UVA radiation, the NO oxidation product nitrite as well as S- or N-nitrosylated proteins may represent an enzyme-independent source for spontaneous NO formation, due to decomposition or photolysis. Indeed, in normal human skin we measure the following contents: 5.1±1.6 µM nitrite, 2.6±1.0 µM S- and 1.3±0.4 µM N-bound NO representing 25-, 370- or 40-fold higher concentrations than found in blood plasma of healthy volunteers. Immunohistochemistry using anti- S-nitrosocysteine-specific antibodies reveals consistent and intensive staining for S-nitroso-proteins all over the dermis, in the cytoplasm of epidermal keratinocytes, and in the stratum corneum. Importantly, we now demonstrate for the first time that UVA-irradiation of healthy skin, at doses used in clinical photo-provocation studies, induces NO formation at concentrations of 8.4±2.7 nM NO/min. NO levels decrease to control values immediately after switching off the UVA source. Testing the biological relevance of this enzyme-independent NO formation we find that UVA-irradiation of skin homogenates, in the RFL-6 reporter cell-assay, leads to a strong increase in cGMP formation, which is inhibited in the presence of the NO-scavenger cPTIO (40 µM). Incubations carried out in the presence of NOS inhibitors demonstrate that an involvement of NOS activities can be excluded. With the discovery of the presented mechanism of enzyme-independent NO production in human skin we open a completely new and fundamental research field in cutaneous biology with a strong relevance for skin physiology and pathophysiology.

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Photodynamic therapy of normal human dermal fibroblasts (NHDF) using several photosensitizers

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Objectives: Photodynamic therapy (PDT) combines photosensitizers absorbing light in the visible spectral region and irradiation with light of corresponding wavelenghts of 662 nm. The sensitivity of HaCaT cells towards PDT with several photosensitizers (Chlorin e6, Photochlorin I, Pheophorbid a) was investigated.Material and Methods: PDT was performed employing 662 nm light and the several photosensitizers using a low energy laser (diode laser Ceralas PDT, 662 nm, 2.5 Watt, gallium-aluminium-arsenide). Biostimulation and bioinhibition were determined in normal human dermal fibroblasts (NHDF) by means of the bioluminesent ATP-assay (LUMIstar Galaxy, BMG Labtechnologies GmbH, Germany), the fluorometric PicoGreen-assay (FLUOstar Galaxy, BMG Labtechnologies GmbH, Germany) and the fluoresence microscopy, respectively. Additionally, the uptake of the photosensitizers was estimated using the microplate fluorometer FLUOstar Galaxy. The phototoxcicity was measured by means of the ATP-assay after irradiation of the NHDF with 0.8 J /cm^2 and 12.5 J /cm^2 of 662 nm irradiation after incubation with serveral photosensitizers in different concentrations (0.006 µM, 0.06 µM, 0.6 µM and 6 µM).Results: Cytotoxcic as well as stimulatory effects could be found for all photosensitizers depending on the their concentrations, the incubation time and the lag time between irradiation and measurement. The following ED_{50}-values were calculated : ED_{50} Photochlorin I (0.015 - 0.140 µM), ED_{50}Pheophorbid a (0.014 - 0.021 µM), ED_{50} Chlorin e6 (0.015 - 0.021µM).Conclusions : The photosensitizers Chlorin e6, Photochlorin I and Pheophorbid a in concentrations higher than 0.6 µM are suitable for photodynamic therapy of human normal dermal fibroblasts using a low energy laser with an energy density of 12.5 J /cm^2.This work was sponsered by the BMBF and biolitec AG Jena.

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Expression of basic fibroblast growth factor (bFGF), stem cell factor (SCF) and endothelin-1 (ET-1) in fibroblasts and keratinocytes exposed to UVA and UVB irradiation

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A dysregulation of cutaneous growth factors leading to uncontrolled proliferation of melanocytes may represent the first step in melanoma development. We have recently shown that an increased expression of bFGF, SCF and Endothelin-3 (ET-3) combined with UVB irradiation can lead to melanoma-like lesions in human skin. We now studied whether UVA or UVB can influence expression of bFGF, SCF, and ET-1 - essential growth factors for melanocytes - in fibroblasts and keratinocytes. Monocultures of foreskin fibroblasts and keratinocytes were irradiated with different doses of UVA (10, 20, 40 J/cm2) and UVB (10, 15, 20 mJ/cm2). Protein expression (supernatant and cell extract) was analyzed by quantitative ELISA.
While bFGF production increased in fibroblasts dose-dependently by UVA (4- to 22-fold) and UVB (8- to 59-fold), it was not significantly changed in keratinocytes by UVA (0.8- to 1.4-fold) and slightly increased by UVB (1.2- to 2.9-fold). These data could be confirmed at the mRNA level by quantitative RT-PCR. SCF protein expression was not changed or decreased in keratinocytes by UVA and not significantly increased by UVB in the supernatants (1.4-fold) and cell extracts (1.4- to 1.9-fold). ET-1 production in keratinocytes was decreased up to 5-fold by UVA and slightly increased by UVB (1.8- to 2.7-fold).
In summary, this is the first time to show that bFGF can be induced in cutaneous fibroblasts by both UVA and UVB at the RNA and protein level. Expression of bFGF, SCF, and ET-1 in keratinocytes was not influenced or decreased by UVA and slightly increased by UVB. These data demonstrate that (1) UVB is more potent than UVA to induce growth factor expression, (2) fibroblasts are more responsive to UV than keratinocytes regarding bFGF production, and (3) the expression of bFGF is more inducible by UV than the expression of SCF and ET-1.

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Perforin granule release from natural killer cells is inhibited dose- and wavelength-dependently by UV-light *in vitro*.

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The pore forming protein perforin (Perf) is employed by $CD8^+$ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to regulate tumor and virus surveillance, T_{H1}/T_{H2} balance, immunoglobulin production and T cells homeostasis. We recently reported about a dose- and wavelength-dependent inhibition of the perforin-granule release from $CD8^{hi+}$ cytotoxic T cells by UV-light. Now, UV-effects on the $CD8^-$ perforin $^+$ NK cells population were investigated.Ficoll-isolated peripheral blood mononuclear cells of 21 healthy donors were irradiated with UVA1 (10, 30, 50, 75, 100 J/cm², n=11), UVA (3, 5, 10, 20 J/cm²; n=15) and UVB (30, 70, 100, 150 mJ/cm²; n=10) and subsequently incubated w/wo PMA and ionomycin for 0, 30, 60, 90, 120 min. Cells were fixed, permeabilized, stained with monoclonal antibodies against Perf and CD8 and analyzed in a FACScan flow cytometer. At time point zero, 20±11% of $CD8^-$ lymphocytes stained $Perf^+$. Setting this value to 100%, the PMA/ionomycin-stimulated Perf release kinetics was determined and was inhibited significantly by UVA, i.e.: 60 min after stimulation, onla 54 ± 23% of unirradiated cells contained perforin as compared to irradiated cells: UVA 10 J/cm² (88±21, $p<0.05$), UVA 20 J/cm² (93±37, $p<0.001$), UVA1 75 J/cm² (81±26, $p<0.05$), UVA1 100 J/cm² (82±27, $p<0.05$). In contrast, UVB had no effect. Taken together, UVA = 10 J/cm² and UVA1 = 75 J/cm² inhibited PMA/ionomycin-stimulated Perf release from $CD8^-$ perforin $^+$ NK cells.

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UVB and UVA activate the Fanconi anemia/BRCA DNA damage response pathway

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The identification of genes defective in the various complementation groups of the cancer-prone, chromosome instability disorder Fanconi anemia (FA) have recently facilitated the elucidation of a novel DNA damage signaling pathway. Central to this pathway is the ubiquitylation of the FANCD2 protein, which depends on the formation of the FA-complex. Upon ubiquitylation, FANCD2 forms nuclear foci with BRCA1, NBS1, MRE11, and Rad50, and activates S-phase DNA repair through homologous recombination and non-homologous DNA end-joining. So far, an activation of this pathway has been described after exposure to DNA-crosslinking agents and ionizing radiation (IR), but not UVA or UVB. FANCD2 is also central to a second DNA damage response pathway, as it is phosphorylated in an ATM-dependent way following IR. In order to elucidate whether these DNA damage response pathways are also activated by UV, we exposed primary human fibroblasts to various doses of UVA (10, 20 and 30 J/cm^2) and UVB (10, 20, 30 mJ/cm^2), and assessed the pathway activation by detecting the FANCD2-S-isoforms (non-ubiquitylated) and FANCD2-L-isoforms (ubiquitylated) by immunoblotting at several time points following irradiation. As early as two hours after irradiation, clear shifts to the (activated) L-isoforms were detected with all doses, and subsequent degradation of FANCD2 after 24 and 48 hours. This clearly indicates that the FA/BRCA pathway is activated by UVA and UVB. Unlike after IR, UV did not result in a shift of the S- and L-isoforms, indicating that UV does not induce the (ATM-dependent) phosphorylation of FANCD2. Since the FA/BRCA pathway activates recombination repair and DNA strand break repair, we hypothesize that both UVA and UVB generate a biologically relevant amount of DNA strand breaks, eg. through oxygen radicals, to require activation of this pathway.

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Assessment of 3 Xeroderma pigmentosum Group C Gene Polymorphisms and Risk of Cutaneous malignant melanoma: A Case-Control Study.

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Individuals with the rare nucleotide excision repair deficiency syndrome xeroderma pigmentosum (XP) are sun-sensitive and exhibit a 1000-fold increased risk for developing skin cancers including malignant melanoma. Inherited polymorphisms of XP genes may, thus, contribute to subtle variations in DNA repair capacity and genetic susceptibility to melanoma. We investigated the role of three newly identified variant alleles of the DNA repair gene XPC in a hospital-based case-control study of 294 caucasian patients from Germany with malignant melanoma and 375 healthy control individuals from the same area matched by age and sex. We confirmed that the PAT+, Intron 11A, and the exon 15C polymorphisms are in linkage disequilibrium. The allele frequencies (cases : controls) were for PAT+ 41.7% : 36.9%, for Intron 11A 41.8% : 37.0%, and for exon 15C 41.3% : 37.3%. The observed genotype distributions matched the expected genotype distributions as predicted by the Hardy-Weinberg theory. The homozygous PAT, Intron 11, and exon 15 genotypes were associated with nonsignificantly increased risks of melanoma: OR 1.527 (95%-CI: 0.967-2.418), OR 1.526 (95%-CI: 0.966-2.414), and OR 1.425 (95%-CI: 0.906-2.246), respectively. Exploratory analyses of subgroups revealed that these genotypes might be associated with significantly increased risks for the development of multiple primary melanomas (n=28), melanomas in individuals with a low number of nevi (n=273), melanomas in individuals older than 60 years (n=100), and melanomas thicker than 1mm (n=126). Our case-control findings support the hypothesis that the PAT+, Intron 11A, exon 15C haplotype may contribute to the risk of developing and progression of malignant melanoma by increasing the rate of an alternatively spliced XPC mRNA isoform that skipped exon 12 and leads to a reduced DNA repair function.

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Infrared-A irradiation disturbs the oxidative status of human skin fibroblasts

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Human skin is increasingly exposed to Infrared-A(IR-A) radiation, which is not only the major part of natural sunlight but also emitted by artificial irradiation devices which are increasingly used for reasons of lifestyle or therapeutic purpose. There is, however, evidence from animal as well as in vitro studies that IR-A irradiation causes premature skin ageing. Accordingly, we have previously shown that IR-A irradiation leads to an induction of matrixmetalloproteinase-1 expression in human dermal fibroblasts via the ERK-MAPKinase signaling pathway. The photobiological mechanisms through which IR-A activates MAPKinases are completely unknown. In the present study we provide evidence that IR-A radiation induced MAPKinase activity and gene expression in human dermal fibroblasts is associated with the generation of an oxidative stress response. Specifically, exposure of fibroblasts to IR-A at doses which were previously shown to activate ERK1/2 and to induce MMP-1 mRNA and protein expression profoundly affected the level of reactive oxygen species (ROS). In particular, the ROS level in the cytoplasm (measured via oxidation of dichlorofluorescein) of irradiated cells is decreased by appr. 20% compared to the sham-irradiated controls, while ROS production in the mitochondria (measured via oxidation of 123-dihydrorhodamine) did not change. With time (0 - 3 h) cytoplasmic ROS level of irradiated cells rised again towards the level of non irradiated cells. When the influence of IR-A radiation on the cellular glutathione (GSH) content and the equilibrium of reduced and oxidized glutathione was assessed utilizing a GSH-Reductase based recycling assay, it could be observed that the equilibrium was shifted towards the oxidized form of glutathione immediately after the irradiation. The total glutathione concentration of irradiated cells was increased 24 h post exposure. Taken together our data for the first time demonstrate that IR-A irradiation has the capacity to affect the oxidative status of mammalian cells. ROS have previously been shown to trigger signaling cascades involving MAPK activation. Ongoing studies are directed at clarifying whether IR-A radiation induced oxidative stress responses are of functional relevance for MAPK activation and gene expression in IR-A-irradiated dermal fibroblasts.

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The platelet-activating factor (PAF) pathway is involved in psoralen and UVA (PUVA)-induced immune suppression

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We hypothesized that PAF (a proinflammatory phospholipid mediator) may be involved in PUVA-induced immune suppression, similar to what occurs after UVB exposure (Walterscheid et al, 2002). The intraperitoneal injection of both a specific PAF receptor antagonist (PCA-4248) or a selective COX-2 inhibitor (SC-236) (0.2 ug/100 ul each; 15 min before UVA treatment) completely abrogated topical 8-MOP and UVA (PUVA)-induced systemic immune suppression in C3H/HeNCr mice in the model of induction of delayed type hypersensitivity to Candida albicans. Interestingly, when phosphatidylcholine (PC) with or without 8-MOP was UVA-irradiated ex vivo in order to produce PAF-like molecules, immune suppression resulted in mice after i.p. injection of both UVA-irradiated, 8-MOP treated or UVA-irradiated, untreated PC solutions. This result contrasted the in vivo situation, in which exposure to UVA alone never resulted in immune suppression. The injection of both PCA-4248 or SC-236 reduced PUVA-induced IL-10 upregulation in the skin and in the serum and anti-IL-10 antibody i.p. injection led to abrogation of PUVA-induced immune suppression. This indicates that activation of the PAF pathway and the downstream production and release of regulatory cytokines are crucial events in PUVA-induced immune suppression. However, the discrepancy in the result of the ex-vivo-to-in-vivo PC experiment compared to the pure in vivo situation suggests that additional molecular mechanisms in addition to PAF generation may be necessary for PUVA-induced immune suppression.

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Ultraviolet A (UVA)-radiation distroys the architecture of plasma membrane -associated rafts in human epidermal keratinocytes (HEKs)

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We have shown that UVA-induced signaling in HEKs is triggered by the release of ceramide from plasma membrane localized sphingomyelin (SM) within 30 minutes post irradiation. Within the plasma membrane of mammalian cells, SM together with cholesterol form microdomains (rafts) which differ according to their microcrystalline structure and fluidity from the remaining plasma membrane and build up platforms, which are involved in membrane trafficking and signal transduction. They contain proteins including caveolin-1 (Cav1), which has been shown to be functionally involved in raft-mediated signal transdcution. Here we asked whether rafts are involved in UVA radiation-induced signaling. Rafts were isolated as Triton X-100 insoluble membranes by several centrifugation steps from HEKs which had been sham-irradiated or exposed to 30 J/cm^2 of UVA radiation. This dose was shown to induce intracellular signaling and gene expression, but not apoptosis or necrosis. Analysis of the lipid composition of the isolated rafts revealed dramatic changes: the SM content decreased to a third of that observed in controls, corroborating our previous observation that UVA radiation-induced ceramide formation results from SM hydrolysis. In addition to SM, the cholesterol of rafts from irradiated cells was decreased by more than 50%. Western blot analysis showed a marked decrease in Cav1 in rafts from irradiated cells, in comparison to untreated controls. In order to follow the fate of Cav1 in UVA irradiated HEKs other cellular compartments were analyzed for Cav1 expression. Neither the cytosolic fraction of HEKs which, in contrast to most other cell types, contain Cav1, nor mitochondria contained increased amounts of Cav1; in fact, in both cell compartments, Cav1 expression was decreased upon UVA treatment. In marked contrast, significantly increased amounts of Cav1 could be detected in the nuclear fraction of irradiated cells. UVA radiation at doses which are functionally relevant for signal transduction and gene expression in HEKs causes a profound disturbance in the architecture of plasma membrane-associated rafts and the translocation of Cav1 from the plasma membrane into the nucleus.

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Effects of UVB irradiation and calcitriol on gene expression of anabolic and catabolic enzymes of the epidermal vitamin D3 pathway

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Earlier investigations in our laboratory (Lehmann et al., JID 117, 1179-1185, 2001) have demonstrated that UVB irradiation of cultured human keratinocytes (optimum at about 300 nm) induces the conversion of 7-dehydrocholesterol (7-DHC) to calcitriol (1a,25-dihydroxyvitamin D3). This pathway involves both the anabolic enzymes CYP27 and CYP1a as well as the catabolic enzyme CYP24, which is a sensitive target gene of calcitriol. Here, we investigated the UVB-induced mRNA expression of CYP27, CYP1a and CYP24 using real time PCR. Interestingly, our results show a strong but transient induction of CYP24 (16-fold of basic level) at 8 h after medium exchange in unirradiated keratinocytes although cultured keratinocytes do not contain detectable amounts of neither 7-DHC nor vitamin D3 and show only traces of calcitriol. That induction peak was followed by a decreasing percentage of cells going through S-phase (FACS). Our experiments show that monochromatic UVB (10 mJ/cm2) had no effect on expression of CYP27 and CYP1a (290 nm - 320 nm) but resulted in a limited induction (4- to 8-fold) of CYP24 at 290 nm and 300 nm (in contrast irradiation at 310 nm and 320 nm led to identical patterns as shown for unirradiated keratinocytes). That increase was not changed by application of 20 mJ/cm2 but was smaller than seen in experiments in which calcitriol synthesis in keratinocytes was primed by preincubation with 7-DHC before UVB irradiation (20 mJ/cm2, 300 nm) (prolonged induction up to 24-fold starting at 8 h). In control experiments where exogenous calcitriol was added to cell culture an immediate and dramatic upregulation of CYP24 (more than 100-fold) was measured. We deduce from these experiments a constitutive expression of CYP1a and CYP27 showing only minimal variation of the mRNA-level. In contrast, CYP24 is, beside its constitutive expression, obviously regulated depending on cell proliferation, UVB irradiation and calcitriol. If confirmed at the protein level, these findings could have important implication for the epidermal calcitriol metabolism and its modulation by UVB radiation in health and disease.

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Influence of 5-Aminolevulinic acid and Photofrin on HUVEC after irradiation *in vitro*

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It is known that a coordinate network of different signal pathways is induced when cells are exposed to reactive oxygen species generated by sub-lethal Photodynamic Therapy (PDT) doses of both photosensitiser (PS) and subsequent irradiation. Human endothelial cells play a crucial role in signal transduction between circulating cells of the immune system and the skin. The aim of the present study was to investigate the effects of PDT on HUVEC, to determine phototoxicity and the expression pattern of cell adhesion molecules (ICAM-1, VCAM-1). HUVEC were incubated with different concentrations (100-1000 µM) of 5-aminolevulinic acid (ALA) inducing porphyrin synthesis and irradiated with an incoherent light source (40 mWcm-2; 24 J/cm-2). Cell viability was measured after 24 h by MTT-assay. No significant decrease of cell viability was detected after incubation and irradiation. However, intracellular accumulation of generated prophyrins was measured in HUVEC after incubation with 5-ALA (1000 µM) by FACS analysis. Detection of porphyrin fluorescence was dependend of the incubation time (0 - 24 h). In contrast, incubation of fibroblasts and keratinocytes with the same 5-ALA concentrations and irradiation resulted in a reduced cell viability of 7%. Moreover, incubation of HUVEC with Photofrin® (0.1 - 0.5 µg/ml) resulted in a significant decrease of cell viability (3%). In separate experiments, HUVEC were incubated with a sub-lethal concentration of 0.1µg/ml Photofrin® and irradiated. FACS-analysis did not show a change of ICAM-1 or VCAM-1 expression thereafter 12 h. In summary, these results show, that phototoxicity of 5-ALA and irradiation is dependent on the cell type. Non phototoxicity of 5-ALA against endothelial cells may explain the less efficacy of ALA-PDT against tumors *in vitro* and *in vivo* relative to that observed for the exogenous PS Photofrin® after PDT treatment of tumors. Sub-lethal phototoxicity of HUVEC initiates no increase of leukocyte adhesion molecules within 12 h after PDT suggesting a restricted response to initiate a physiological cascade regulation after PDT.

P153

Systemically administered ascorbic acid and d-alpha-tocopherol reduce DNA damage in vivo

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UVB irradiation is known to cause DNA damage, particularly cyclobutane pyrimidine dimers and (6-4) photoproducts, which induce mutations in epidermal cells and may lead to skin cancer. The use of sunscreens reduces not only UV-induced erythema, but also the development of thymine dimers (TD) in human skin. Antioxidants like ascorbic acid and d-alpha-tocopherol (AAAT) have been found to decrease skin sensitivity to UV irradiation in humans. Recently we reported that systemic administration of antioxidants results in extension of the UV-induced cell cycle block and thereby could allow for more time to repair DNA damage. But as there is yet no clear evidence for actual protection against UV-induced DNA damage by AAAT, we performed further studies.
17 individuals took orally a daily dose of 2 g ascorbic acid and 1000 IU d-alpha-tocopherol for 3 months. Biopsies were taken from UVB-irradiated (twice the minimal erythema dose) and unirradiated skin before and after 90 days of AAAT administration. TD in epidermal cells were visualized in sections of paraffin-embedded tissue by a monoclonal antibody and the labelled streptavidin biotin method. Immunohistochemical staining for Cyclooxygenase-2 (COX-2) was done in the same manner. In unirradiated skin no TD-positive cells were found. Following UV-exposure the median number of TD-positive cells at baseline was 82.0 cells/mm epidermis, during administration of AAAT for 90 days it was decreased to 42.8 cells/mm ($p < 0.002$). Parallel to this, a decrease of COX-2 – an enzyme known to be associated with carcinogenesis – was observed (10.2 vs. 6.9 cells/mm; $p < 0.01$). These findings suggest that administration of AAAT has a protective effect against UV-induced DNA damage. These antioxidants might be useful in prophylactic measures against photocarcinogenesis.

P154

Tumor therapy with ICG-mediated phototherapy: comparison between continuous wave and pulsed diode laser *in vivo*

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The delivery of drugs and immunoconjugates by the heterogenous tumor vascular system to tumor cells is limited. New therapeutic strategies targeting the existing tumor blood vessels (vascular targeting) have emerged. Phototherapy using the clinically approved dye indocyanine green (ICG) is one of these new therapeutic approaches. In this study the efficacy of continuous wave and pulsed diode laser used for irradiation of ICG was compared.Following determination of the fluorescence kinetics for optimal irradiation time the efficacy was evaluated by measuring the tumor volume of amelanotic melanoma cells (A-mel-3) grown in the back of Syrian golden hamsters (n = 62) up to 28 days following therapy. Tumor cells ($5x10^6$) were implanted subcutaneously under ketamine/xylazine anaesthesia. After 4 days the tumors had grown to a mean volume of approximately 100 mm^3. Permanent indwelling catheters were inserted. ICG was applied (0, 2 or 4 mg kg^{-1} b.w.; ICG-Pulsion, Munich, Germany) and laser irradiation was performed ($?_{em}$ = 805 nm; continuous wave diode laser: 100 J cm^{-2}; 0.5 W, pulsed diode laser: 3.2, 10.6 or 32 J cm^{-2}; 0.5 W; pulse duration 3, 10 or 30 ms) directly thereafter. For histological staining (NBTC, CD31, TUNEL) transparent dorsal skinfold chambers were implanted on hamsters and irradiated as aforementioned (n=30). Tissue was taken 24 h following irradiation. Only continuous wave laser irradiation resulted in complete remission of subcutaneous implanted tumors (6/6 following 4 mg kg^{-1} b.w. ICG respect. 3/6 tumors following 2 mg kg^{-1} b.w. ICG). Pulsed diode laser irradiation resulted in complete progression of all tumors (n=38), although the tumor growth curve showed a significant tumor growth delay within the first five days following treatment with a pulse duration of 30 ms and an ICG concentration of 2 or 4 mg kg^{-1} b.w. ICG. Immunohistochemistry showed unspecific coagulation necrosis in the upper tissue layers following continuous wave laser irradiation. Following pulsed diode laser irradiation selective photothermolysis of the blood vessels could be shown, however an unselective damage in the collateral tissue is needed to yield a complete tumor remission as affirmed in the tumor growth curves.

P155

8-Isoprostane as a dose-related biomarker of acute photooxidative UVB-damage in vivo

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UVB radiation is a mainstay of treatment in photodermatology and an important propagator of photochemical effects in human skin linked with the evolution of skin cancer and skin aging, many of those being due to photooxidative stress.
The doses of UVB acquired are up to today measured in in the physical unit Joule and in fractions of the minimal erythema dose (MED) as a relative unit related to a visible UVB-mediated effect on the skin. In order to develop specific biomarkers for individual photooxidative UVB effects in the skin and to be able to relate applied UVB doses to effects of that on a molecular level we have developed a personal dosimetry concept involving a biological as well as a radiophysical tracer. We then surveilled healthy volunteers in a dose-kinetic study with our dosimeters. After exposure to UVB in a range of 25-100 kJ/m^2 we took biopsies 30 minutes later and looked at well established end parameters of oxidative DNA damage (8-OHDG), lipid peroxidation (8-isoprostane) and angiogenesis (VEGF) in an immunohistochemical analysis with densitometric quantification.
Our analysis revealed 8-Isoprostane as a first photooxidative dose-dependent UVB damage parameter. 8-OHDG was also significantly induced by UVB doses above 50 kJ/m^2, but only in a non dose-related on/off pattern. VEGF was not found to be induced in the given setting, probably because the time window chosen was to short for this marker to raise significantly.
Our study demonstrates for the first time 8-Isoprostane as a dose-related photooxidative UVB effect in vivo. The future perspective of a personal dosimetry for surveillance of UVB exposures with a direct link to photobiological effects in terms of a dosimeter dose-biological effect equation has come a step closer.

P156

Supplementing the skin barrier with vitamin E by rinse-off products protects from photo-oxidative damage of skin surface lipids

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Vitamin E is generally regarded as the most important lipid soluble antioxidant in human tissues. Several studies demonstrated the protective effects of topically applied a-tocopherol leave-on formulations against immunosuppression, DNA damage, and carcinogenesis. Only little is known about the penetration characteristics and efficacy of a-tocopherol in rinse-off products. Previously, we reported that the sebum lipid photooxidation product squalene monohydroperoxide (SqmOOH) is induced by photo-oxidation in vivo [1]. Here, we demonstrate that SqmOOH penetrates into skin. The further objectives of this study were to investigate whether the use of an a-tocopherol enriched rinse-off product provides effective deposition of a-tocopherol and photoprotection of skin surface lipids. To test this, forearm skin of volunteers was washed either with an a-tocopherol enriched rinse-off product (TP) or with an a-tocopherol free vehicle control (CP; contralateral arm) using a standardized wash protocol (n=13). Thereafter, skin surface lipids were extracted with pure ethanol at t= 0h or 24h. Additionally, some volunteers were subjected to a single irradiation of their forearms with low-dose UVA (8 J/cm2) before lipid extraction (n=6). For testing of SqmOOH penetration, stratum corneum extractions were performed in various layers after a 2h SqmOOH application period (n=8). Skin lipid extracts were analyzed by HPLC using electrochemical detection for vitamin E and UV-detection for squalene and SqmOOH. Remarkably, the results of this in vivo study demonstrate that 1) CP treatment lowers, TP treatment strongly increases ?-tocopherol levels of skin surface lipids 2) increased vitamin E deposition effects were maintained for a period of at least 24 h, and 3) TP treatment provided significant photoprotection of the skin surface lipid squalene. 4) In untreated skin SqmOOH penetrates skin in vivo. Thus, the use of a-tocopherol enriched rinse-off products may help to maintain the integrity of the environmentally exposed skin barrier.

[1] Ekanayake Mudiyanselage S, Hamburger M, Elsner P, Thiele JJ; J. Invest. Dermatol., Vol 120, (6), 915-922

P157

Targeted Inactivation of Vegf Sensitizes Mouse Skin to UVB-Induced Cutaneous Photo-Damage

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Exposure of skin to sunlight results in erythema, dilation of dermal blood vessels and vascular hyperpermeability, suggesting that these changes in the dermal vasculature are necessary components of the protective response to UV-induced photodamage. Vascular Endothelial Growth Factor (VEGF) is one of several pro-angiogenic factors that are induced in skin after UVB irradiation, and epidermal keratinocytes (KC) are the major source of VEGF in skin. Using the Cre/LoxP system under the control of the keratin5 promoter, we have generated mice in which VEGF has been inactivated in epidermal KC (VEGF-A? k5-cre/ ? k5-cre), and used these animals to study the contribution of KC-derived VEGF to acute and chronic UVB-induced photodamage. We found that these mice developed burn-like lesions after a single UVB irradiation, at a dose at which the control mice were unaffected. Microscopic examination of the irradiated skin revealed massive inflammation, with loss of the epidermis in the mutant but not in the control mice, and impared vascularization in the upper dermis of mutant mice. Double immunofluorescent stains for CD31 and active caspase 3 revealed increased numbers of apoptotic endothelial cells in UVB irradiated mutant mice. Differential immunofluorescent stains for CD31 and Ki67 demostrated reduced numbers of proliferating endothelial cells in mutant mice. We also performed quantitative analysis of cutaneous blood vessels of mice after 10 weeks of UVB irradiation. Cutaneous vascularization was greatly diminished in mutant mice with a prominent effect on large-sized vessels.In the absence of functional VEGF in epidermal KC, the skin is extremely sensitive to UVB-induced photo-damage, characterized by a reduction of subepidermal blood vessels.Whether VEGF protects keratinocytes directly or indirectly via modulation of blood vessel density is still under investigation.

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Single PUVA treatment of human dermal fibroblasts results in longterm enhanced generation of reactive oxygen species being causally responsible for subsequent telomere erosion and premature replicative senescence

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Premature aging of the skin is a prominent side-effect of psoralen photoactivation. We have shown previously that a single non-toxic exposure of human dermal fibroblasts to psoralen photoactivation (PUVA) resulted in a long-term yet reversible growth arrest with reduction of the overall life span. In order to analyze the underlying mechanism, we addressed the questions (1) whether enhanced and persistent oxidative stress is generated post PUVA-treatment, (2) if so, whether it contributes to telomere erosion and (3) whether ROS-dependent telomere erosion is causally responsible for the early onset of replicative senescence of fibroblasts post PUVA treatment. Using the ROS sensitive bioprobe dichlorodihydrofluorescein diacetate (DCF), we found that PUVA-treated fibroblasts revealed an up to 20-fold increased production of reactive oxygen species (ROS) at all studied time points (1 day, 1 week and 6 weeks) after PUVA treatment. The antioxidant N-acetylcysteine, lipoic acid and tocopherol effectively prevented PUVA-induced enhanced ROS production, almost completely prevented telomere erosion and rescued the premature replicative senescence. Using fibroblasts stably transfected with a plasmid driving expression of the catalytic subunit of telomerase (hTERT), which is known to maintain telomere length, we did not observe any decrease in the proliferative capacity after PUVA treatment compared to mock-transfected, PUVA-treated fibroblasts. Collectively, our data indicate that a single PUVA treatment result in persistently enhanced ROS production with profound decrease in telomere length being responsible for the early onset of replicative senescence. In fact, fibroblasts grown from biopsies of PUVA-treated skin show a significantly reduced lifespan compared to fibroblasts derived from non-exposed skin of the same individual, indicating that our in vitro-finding may have in vivo relevance. The cellular origin of PUVA-enhanced ROS production requires further investigation.

P159

Defects in the MHC class I antigen processing pathway: Association with clinical outcome of patients with squamous cell carcinoma.

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MHC class I surface expression is frequently altered in head and neck cancer and represents a strategy to escape from immunosurveillance. To determine the underlying mechanism(s) of deficient MHC class I surface expression, tumor biopsies and cell lines from primary, recurrent or metastatic head and neck tumors were analyzed for expression and for function of various antigen presentation-related genes, including the interferon-? inducible proteasome subunits, the peptide transporter TAP, the chaperone tapasin and MHC class I antigens. A high frequency of downregulation or even loss of LMP2, LMP7, TAP1 and tapasin expression was detected in tumor lesions and cell lines from head and neck cancer patients with deficient MHC class I expression which could be partly corrected by interferon-? treatment. In MHC class I promoter assays it could be shown, that reduced MHC class I expression is in some extent conveyed by decreased transcriptional activity. In addition, for one cell line the methylation of the class II transactivator (CIITA) promoter IV, could be made responsible for reduced MHC class I and II expression. Immunohistochemical staining of tumor tissue of these patients for five markers of the antigen processing machinery, MHC I, TAP1, TAP2, LMP2 and LMP7, was found to predict overall survival and the level of LMP7 expression was significantly associated with disease recurrent at two years. Thus, our results suggest that defects in expression of antigen processing components in the tumor might serve as valuable prognostic factors for this disease and provides insights into molecular mechanisms underlying APM deficiencies in HNC and, for the first time, links their presence to patient survival.

P160

Inhibition of uPAR by a small interfering RNA in melanoma cells

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The plasminogen activator system plays a key role in invasion and metastasis of several human malignancies. In melanoma, the expression of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR) is increased in advanced disease stages and correlates positively with the invasive potential of melanoma cells. Furthermore, interference with uPA/uPAR expression or function reduces invasion and/or metastasis in animal models of human tumors, including melanoma. For further characterization of the role of the plasminogen activator system in melanoma, we have developed a small interfering RNA (siRNA) for uPAR as a molecular tool and as a potential anti-metastatic strategy. A suitable siRNA target sequence was identified in the uPAR mRNA corresponding to a portion of the 4th exon, and an siRNA was designed. Effective transfection into the metastatic melanoma cell line 1205Lu was established using an FITC-labeled siRNA and flow cytometry. Basal uPAR surface expression as assessed by flow cytometry was reduced 24 h after transfection of the uPAR siRNA, whereas a sequence-controlled siRNA had no effect. Additionally, PMA induction of uPAR was prevented by siRNA resulting in a 75% reduction of uPAR expression compared to PMA-induced cells treated with the control siRNA. The inhibitory effect was confirmed at the mRNA level by quantitative RT-PCR showing an 80% reduction of basal and a 70% reduction of PMA-induced uPAR mRNA expression. Taken together, we have designed an uPAR siRNA that effectively and sequence-specifically inhibits uPAR expression in a melanoma cell line. This siRNA will be used to further investigate the role of uPAR in melanoma invasion and metastasis and might lead to the development of a new molecular therapeutic strategy for melanoma.

P161

Chip technology in melanoma: identification of new targets for therapy

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One important application of DNA microarray technology is the simultaneous analysis of gene expression of different mRNA species. Comparison of mRNA patterns of diseased and healthy tissues may help to understand the pathogenesis of a given disorder. Identified dysregulated genes in cancer tissue may function as new molecular markers for diagnosis or prognosis and may ideally serve as more targes for therapy.Using membrane cDNA arrays technology we analyzed gene expression of human melanoma, one of the most agrgressive types of cancer with a high metastatic potential and with increasing incidence worldwide. To account for the heterogeneity of different tumors we analyzed mRNA expression from 10 different melanoma metastases from 10 individual. An abundance of genes were dysregulated (up/down) which involved for examlpe in apoptosis genes like FAST, TFAR 15, GRB10, or angiogenesis molecules such as VEGF. Here, we focus our description on the JAK/STAT signaling pathway which is involved in cell proliferation, cell differentiation and apoptosis. Stat-induced inhibitor 2 (SSI-2) is upregulated in human primary melanocytes in comparison to human melanoma metastases. SSI-2 is a member of a protein family, that inhibits cytokine responses and activation of ´signal transducers and activators of transcription´ (STAT).Therefore, we investigated the protein expression of Stat3 and Stat5 via Western blot analysis and observed a constitutive level of these proteins in primary melanocytes as well as in malignant melanoma metastases. In contrast, the phospho-Stat3 and phospho-Stat5 was weakly expressed in primary melanocytes and upregulated in melanoma metastases. The upregulation of SSI-2 in primary melanocytes seemed to negatively regulate cytokine signal transduction, Stat3, and Stat5 activation negatively. This leads to normal proliferation and differentation of melanocytes. In contrast to this lack of SSI-2, the upregulation of phospho-Stat3, and phosph-Stat5 in melanoma metastses seems to be involved in dysegulation of proliferation. Therefore, targeting of Stat3, (e.g. by dominant negative Stat3ß and Stat5 signaling may provide a potential therapeutic strategy for malignant melanoma.

P162

Selective inhibition of a mutated BRAF allele in melanoma cells by RNA interference

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BRAF is a serine/threonine kinase that transduces growth signals from RAS to MEK1/2 in the MAP kinase pathway. A mutation in exon 15 of the BRAF gene (V599E) is frequently found in melanomas and nevi indicating that there is a strong selection process for this mutation in melanocytic tumors. The V599E mutation leads to a modified BRAF protein that constitutively activates MAP kinase signaling thereby stimulating tumor growth. Hence, selective inhibition of mutated BRAF may be useful as an antiproliferative treatment of melanoma. In an attempt to develop a BRAF inhibition strategy, we have designed a small interfering RNA (siRNA) for V599E-mutated BRAF. Effective transfection into melanoma cell lines was established using an FITC-labeled siRNA and flow cytometry. Effects of siRNA on BRAF expression were assessed by quantitative RT-PCR that detects both wild type and V599E-mutated mRNA. When the mBRAF siRNA was transfected into the metastatic melanoma cell line 1205Lu that has one V599E-mutated and one wild type allele, BRAF-mRNA was reduced by 75% 24 h after transfection, whereas a sequence-controlled siRNA had no effect. This observation suggests specific siRNA-mediated knock down of the V599E-mutated mRNA, whereas mRNA transcribed from the wild type allele may not be affected and account for low residual BRAF expression. In line with this, BRAF-mRNA was neither reduced by mBRAF siRNA nor by control siRNA in the radial growth phase melanoma cell line SBcl2 that carries two wild type alleles. Taken together, the data demonstrate the selective targeting of V599E-mutated BRAF, but not wild type, BRAF by the designed siRNA. Selective siRNA-mediated silencing of mutated BRAF may lead to a new molecular antiproliferative strategy for melanoma.

P163

New CTCL-associated genes identified by SSH-screening and analyzed for serological recognition

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In the advanced stage, cutaneous T-cell lymphoma (CTCL) is very difficult to treat, consequently, immunotherapeutical strategies are developed. The knowledge of antigens associated to CTCL is essential as prerequisite for early detection, diagnosis, prognosis and for successful immunotherapies of CTCL.
To identify new antigens, we used suppressive subtraction hybridisation (SSH) in combination with DNA hybridization analysis of recombinantly expressed cDNA libraries (HYREX) approach. Differentially expressed cDNA in CTCL compared to PBMC of healthy volunteers was enriched, labelled and used as probe to screen a phage library.
We could identify 37 CTCL related cDNA phage clones, representing both, novel and known genes. As a first hint for their value as prognostic marker or therapeutic target, the identified clones were analyzed in terms of their sequence homologies to known genes, their expression profile and sero-reactivity of patients and healthy donors against these proteins.
While it might be expected, that inflammatory markers and genes encoding proteins involved in increased metabolism and cell duplication were identified, we noted that the majority of the detected differentially expressed genes have been reported to be associated with cancer or are involved in tumorigenesis or tumor progression. During serological test we found that 10 of these clones were recognized by at least one CTCL serum but not by sera of healthy donors. Expression analysis of cancer associated genes was first performed using virtual northern blotting on the basis of the homologous EST database entries. All genes, which were positive in less than 10 different normal tissues types, were selected for RT-PCR analysis resulting in moderate to high frequencies of positive control tissues.
The high percentage of cancer-associated clones contrasts the RT-PCR results and implicates the necessity for further analysis on protein level. We conclude, that the identified panel of CTCL associated genes might harvest several interesting genes possibly involved in CTCL pathogenesis and might be suitable as diagnostic/therapeutic targets.

P164

Induction of serum antibodies against identified tumor antigens in melanoma patients during vaccination with autologous tumor cells

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While the existence of a humoral response against tumor-associated antigens is well appreciated, a systematic analysis of its possible induction by the tumor is still missing. We compared the specific IgG response of stage IV melanoma patients during vaccination. Patients had been treated within two clinical trials with autologous tumor cells gene-modified for IL-7 or IL-12 (Br J Cancer, 1998, 77: 1907-16; Gene Ther, 1998, 5: 481-90). A panel of 27 tumor-associated antigens (HD-MM-01 to HD-MM-27) was isolated by a SEREX screening of a testis cDNA library using a pool of five sera from patients after vaccination. All antigens were retested with individual sera of 12 patients both pre and post vaccination. Against 18 antigens a serological response was induced during vaccination. Remarkably, induction was detected only in patients included in the screening pool. The low overlap between sero-reactivity of the 12 patients suggested a very individualized immunological reaction. Two of the 5 sera included into the screening pool exhibited a high frequency of induced humoral responses. The same patients had been shown to have a high Karnovsky index and had generated lytic cytotoxic T cells against the tumor. Besides two known cancer-germline genes (SCP-1 and PLU-1), the other isolated antigens were expressed in a non-tumor-specific fashion as analyzed by virtual Northern blot and/or RT-PCR. The properties of homologues to several of the identified tumor-antigens, especially PLU-1, SCP-1, DNEL2, CLOCK, and PIASx-alpha, suggest further investigation of their possible function in malignant melanoma. We conclude that a strong humoral response against tumor-associated antigens is inducible by tumor cells and that this response is very individual.

P165

Histonedeacetylation and calpain activity modulate Vitamin D-induced growth inhibition in melanoma cell lines.

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Increasing evidence points at an important function of the skin vitamin D system for tumorigenesis and progression of malignant melanoma. We have characterized the vitamin D system in melanoma cell lines, detecting strong RNA expression of vitamin D receptor (VDR), 25-hydroxyvitamin D-1a-hydroxylase (1aOHase), vitamin D-25-hydroxylase (25OHase) and 1,25-dihydroxyvitamin D-24-hydroxylase (24OHase) in various melanoma cell lines (MEWO, SKMEL28, BU47HOM) using real time PCR (LightCycler) and specific hybridisation probes. We then have analyzed effects of 1,25-dihydroxyvitamin D_3 and analogs (EB 1089, MC 1288) on proliferation and apoptosis in various melanoma cell lines (BUHOM, MEWO, SKMEL) *in vitro*. Using a tetrazolium salt (WST-1) based colorimetric assay, we detected dose-dependent inhibition of cell growth (up to 40%) induced by calcitriol or its analogs. Treatment of melanoma cells with calcitriol resulted in decreased immunoreactivity of proliferation markers including Ki-67 and PCNA. Flow cytometry experiments (bcl-2, bcl-xl, bcl-xs, bcl-x, bax, CD95) and analysis of annexin Pi expression revealed no induction of apoptosis by calcitriol (10^{-7} M) or its analogs, while increased levels of bcl-2 protein were detected. Additionally, we show modulation of vitamin D-induced inhibition of cell proliferation by was analysed using calpain inhibitors I and II (Calbiochem) as well as by inhibitors of histonedeacetylation (sodium butyrate, trichostatin A). In conclusion our findings indicate that (i) vitamin D analogs suppress proliferation but do not induce apoptosis in melanoma cell lines (ii) new calcitriol analogs exerting less systemic side effects may be interesting candidates for the treatment of metastasizing malignant melanoma.

P166

Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis

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The Ras/Raf signaling pathway is contributing to several hallmarks of cancer. Hence, its constitutive activation has been suggested to impact the clinical course of the tumor. To address this notion, we analyzed tumor DNA from 114 primary cutaneous melanomas and of 86 metastatic lesions obtained from 174 patients for mutations in BRaf (exons 15 and 11) and NRas (exons 1 and 2) by direct sequencing of PCR products and correlated these results with the clinical course. This analysis revealed that in 57,5 % of the melanoma lesions either BRaf or NRas were mutated with a higher incidence in metastatic (66,3 %) than in primary lesions (50,9 %). Although the majority of BRaf mutations affected codon 599, almost 20 % of the 599 mutations were different from the well-described exchange from valine to glutamic acid. These mutations (V599R, V599K and V599E) also displayed an increased kinase and transforming activity. With respect to the correlation of mutational status and clinical course, the presence of BRaf/NRas mutations in primary tumors did not negatively impact progression free or overall survival. In contrast, however, for metastatic lesions the presence of *BRAF/NRAS* mutations was associated with a significantly poorer prognosis, i.e. a shortened survival.

P167

Increased Incidence of Squamous Cell Carcinomas in Mastomys natalensis Papillomavirus E6 Transgenic Mice during Experimental Skin Carcinogenesis

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Papillomaviruses cause certain forms of human cancers, most notably carcinomas of the uterine cervix. In contrast to the well established involvement of papillomavirus infection in the etiology of cervical carcinomas and in carcinomas of a rare hereditary condition, Epidermodysplasia verruciformis, a causative role of cutaneous human papillomavirus types in the development of nonmelanoma skin cancer has not been proven up to now. In order to better understand the function of individual genes of cutaneous papillomavirus types, we generated transgenic mice carrying the oncogene E6 of the Mastomys natalensis papillomavirus (MnPV), causing keratoacanthomas of the skin in its natural host. In the present study we demonstrate that MnPV E6 transgenic mice develop squamous cell carcinomas to nearly 100% under conditions of experimental two-stage skin carcinogenesis, in comparison to 10% in the non-transgenic littermates ($p<0.0001$). Therefore we conclude that the MnPV E6 transgene positively affects malignant progression of chemically-induced tumors. Whereas the H-ras gene was mutated at codon 61 in benign as well as malignant mouse skin tumors induced by the combined treatment of 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), tumors expressing high levels of MnPV E6 mostly reveal non-mutated H-ras alleles. These results support the notion that MnPV E6 is able to substitute for an activated H-ras gene.

P168

The Expression of Psoriasin (S100A7) in Premalignancies and Epithelial Skin Tumors is not Dependent on Differentiation and Inflammation

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Psoriasin is a calcium-binding S100-protein and was originally identified as an upregulated protein in psoriatic keratinocytes. Our own experiments identified psoriasin as a major *E. coli* killing antimicrobial protein which is inducible in primary keratinocytes by differentiation, proinflammatory cytokines and contact with microorganisms. Recently, psoriasin was shown to have elevated expression in *in situ* ductal breast carcinoma as well as in bladder squamous cell carcinoma but the function of this protein remained unclear. Preciding studies from our group showed a higher expression of psoriasin m-RNA in epithelial skin tumors compared to healthy skin. This study was performed to get insight into the mechanism and function of psoriasin expression in human epithelial skin tumors.
After isolation of total-RNA from punch-biopsies realtime-RT-PCR-experiments were performed with intron-spanning primer pairs for psoriasin, involucrin (as marker for epithelial differentiation) and interleukin-8 (as marker for inflammation).
In all biopsies from patients with premalignancies (n=6), basal cell carcinomas (n=17) and squamous cell carcinomas (n=11) a significant higher expression of psoriasin compared to the control group (n=10) was seen. Interestingly, non-lesional skin from all tumor-patients shows a higher expression than skin from healthy patients. In all tumor-entities involucrin showed no significant induction of m-RNA-expression, whereas interleukin-8 was significantly higher expressed in the group of squamous cell carcinomas. In this group of tumors there was no correlation between elevated psoriasin mRNA-expression and the expression of interleukin-8 or involucrin.
In summary, our experiments show that psoriasin-mRNA-expression in human skin tumors is not dependent on differentiation and inflammation.

P169

Expression of the transcriptional repressor Snail and the cell-cell-adhesion molecule N-cadherin are involved in regulation of MAPK activity in melanoma cells

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Crosstalk between melanocytes and keratinocytes is important in human epidermis. It is known that a normal melanocytic phenotype and controlled proliferation of melanocytes is strictly regulated by keratinocytes via E-cadherin. Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression and the upregulation of N-cadherin. This leads to the loss of regulatory dominance by keratinocytes. Further, melanoma cells can now get into contact with fibroblasts via N-cadherin and induce e.g. MMP expression.
We previously could show that loss of E-Cadherin in melanoma cell lines involves upregulation of the transcriptional repressor Snail. In comparison to primary human melanocytes, where Snail expression was not detected by RT-PCR, significant expression was found in melanoma cell lines. Consistently, transient and stable transfection of an antisense snail construct induced re-expression of E-cadherin in melanoma cell lines. N-cadherin, which is strongly expressed in melanoma cells was shown to be downregulated analyzing this cell clones. We further analyzed the MAP kinase signaling pathway which is known to be permanently active in melanoma cells. Re-expression of E-cadherin in the melanoma cells was associated with reduced MAPK activity.
In summary, we conclude that activation of Snail expression plays an important role in regulating contacts of melanoma cells to their environment and therefore, tumorigenesis of malignant melanomas in vivo.

P170

Analysis of cell signalling by MIA in active detachment of melanoma cells

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Analysing the function of MIA, a protein strongly expressed in melanoma cells but not in melanocytes, we determined strong inhibition of attachment by MIA when melanoma cells are plated onto fibronectin (Fn), laminin (Ln) and tenascin (Tn). No inhibition of attachment to collagen type I, II and IV, HSPG and vitronectin was observed. Further immunoassays showed that MIA binds to the matrix proteins Fn, Ln and Tn and thereby masks the binding sites of integrins to these ECM components. Antibodies against the active center of the integrins a4ß1 and a5ß1 also crossreact with MIA, suggesting a structural homology between MIA and the active integrin binding pocket.
Many studies have shown that changes in attachment of cells do result in changes of intracellular signalling. Therefore we analysed whether MIA had an influence on the major MAPKinase pathways. MAPK activity assays hinted at regulatory processes of MIA on MAPK- pathways in melanoma cells . Through further investigation we showed that increasing time of MIA incubation of melanoma cells decreases Erk- phosphorylation. Other important signalling molecules like p38, paxillin and SHC do not seem to be involved in MIA signalling. To determine if MIA regulates ERK-phosphorylation specifically and not only via signals based on cell detachement we incubated melanoma cells with RGD peptides. The results revieled no specific regulation of ERKs by incubation with RGD peptides.
Taken together our results show that inhibition of adhesion by MIA leads to inhibition of specific intracellular signalling pathways.

P171

Expression of Sno suppresses BMP-Smad signaling in melanoma cells

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It is well known that TGF beta is a multifunctional growth factor regulating many aspects of cellular processes such as proliferation, differentiation, adhesion and apoptosis. This complex signaling pathway is mediated via different Smad proteins regulating gene expression. Melanoma cells produce TGF beta 1, 2 and 3 themselves subsequently leading to activation of the TGF beta/Smad signaling pathway. We could show, that besides TGF beta also BMPs are expressed by melanoma cells and activate Smad signaling. In contrast to TGF beta which signals via Smad 2 and 3, BMPs activate the BMP-Smad-pathway targeting Smad 1 and 5. Interestingly, the TGF beta and BMP expression does not result in inhibition of cell proliferation. The aim of our study was to analyze the mechanisms melanoma cells use to escape the growth control mediated by Smads. Recent publications suggested that overexpression of the smad-co-repressor Ski is responsible for resistance to TGF beta-antiproliferation signals.
Since we could only detect weak or no expression of Ski in all but one melanoma cell lines analyzed we concentrated on Sno, a family member of Ski, and found strong expression of Sno on mRNA and protein level. Further, repression of Sno via transient transfection of an antisense sno expression plasmid restored function of the BMP-Smad pathway in melanoma cells.
These results implicate an important role of the co-repressor Sno in melanoma progression enabling the cells to uncontrolled proliferation.

P172

Characterisation of ets-1 expression in malignant melanoma

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The transcription factor ets-1 regulates the expression of several angiogenic factors and of proteins involved in extracellular matrix organisation. It was shown to be implicated in disease progression in some kinds of tumors predominantly leukaemia. In the present study, we analysed ets-1 expression and functional importance in malignant melanomas. We could clearly show in vitro and in vivo that expression of ets-1 is strongly upregulated comparing malignant melanoma cells to primary melanocytes. Assays to analyse DNA-binding and transcriptional activity of ets-1 further documented strong ets-1 activity in melanoma cells. Using an antisense attempt, expression and activity of ets-1 was reduced in the melanoma cell line Mel Im. Consequently this led to reduced expression of MMP1, MMP3, uPA and integrin beta 3, all known target genes of ets-1. Additionally, the invasive potential of the cells measured in a Boyden Chamber model was reduced up to 70%. This seems - at least in part - to be attributable to the role of ets-1 in transcriptional regulation of factors involved in invasion on melanoma cells. We therefore conclude that upregulation of ets-1 expression during melanoma development contributes to the malignant phenotype.

P173

Tumor cell induced expression of matrix metalloproteinase-2 in stromal cells via extracellular matrix metalloproteinase inducer (EMMPRIN; CD147)

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Matrix metalloproteinases (MMPs) are essential for tumor progression, invasion and metastases formation. Expression of these proteinases is not restricted to the tumor cells themselves, but also found in normal stromal cells. Extracellular matrix metalloproteinase inducer (EMMPRIN; CD 147) on the surface of tumor cells has been suggested to stimulate MMP production in tumor stromal cells. To scrutinize this hypothesis we established a slowly growing, syngeneic tumor model using the B16-melanoma cell line B78D14. *In vitro* analysis demonstrated that B78D14 cells secreted MMP-2, MT1-MMP and to a lesser degree MMP-9; in addition they expressed both MT1-MMP and EMMPRIN on their surface. Coculture experiments revealed a tumor cell dependent production of MMP-2 in normal murine fibroblasts. *In vivo* experiments confirmed this notion. In subcutaneous tumors MMP-2 expression was predominantly present at the tumor-stroma border suggesting stromal cells as primary source for this protease *in vivo*. Indeed, double staining experiments and *in situ* zymography confirmed that tumor adjacent stromal cells at the invasive front expressed MMP-2; moreover only at this site activated MMP-2 was detectable. Notably, in a experimental pulmonary metastases model, which is characterized by an absent EMMPRIN expression on tumor cells neither tumor nor stromal cells expressed MMP-2. Hence, EMMPRIN expression on the tumor cells seems mandatory for the induction of MMP-2.

P174

A mutation in the osteopontin promoter causes different responsiveness to upstream signalling pathways in melanoma cells

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Melanoma progression is a multistep process involving a variety of molecules. In the present report we used the oligonucleotide microarray technique to analyse the gene expression pattern of 12.000 genes in 6 pairs of primary melanomas and metastases (each pair from the same patient). Biostatistical analysis identified osteopontin as a new extracellular matrix molecule differentially expressed in primary melanomas versus metastases. Genetic analyses of melanoma cell lines of different aggressiveness revealed a mutation in the osteopontin promoter present only in highly aggressive cell lines. This mutation lies within a c-myb transcription factor binding site. Further promoter studies were performed by in vitro luciferase assays in order to elucidate whether this mutation impacts on the transcriptional regulation of osteopontin. Interestingly, in co-transfection studies we found that members of the family of mitogen-activated protein kinases exerted opposite effects on the osteopontin promoter depending on the sequence of the c-myb binding motif. In particular, activation of the MKK6/p38 pathway led to a down-regulation of the mutated and slight up-regulation of the wildtype promoter. Thus, specific activation of the MKK6/p38 pathway might serve as an inhibitor mechanism of osteopontin production in the presence of a mutated promoter. In conclusion, we were able to identify a new factor in malignant melanoma with different expression during tumor progression. We further identified activated MKK6/p38 pathway as a putative inhibitor of osteopontin production. The presented findings provide a new target structure for future therapeutic approaches in malignant melanoma.

P175

Inhibition of Migratory Inhibitory Protein (MIA) leads to molecular and phenotypic changes

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Early molecular events in tumor development of malignant melanoma are still largely unknown. Recent evidence reveals that the secreted protein MIA (melanoma inhibitory activity) is highly expressed in malignant melanomas and associated with tumor progression in vivo. We therefore investigated the role of MIA in melanoma cells by inhibiting MIA expression in the human melanoma cell line HMB2 via stable antisense MIA cDNA transfection and analyzed the cell clones. MIA-deficient cell clones showed changes in cell morphology like size and dentritic protrusion. Further, in monolayer culture and spheroid system enhanced cell-cell-contacts were formed. Interestingly re-induction of pigment synthesis in comparison to the amelanotic parental cell line HMB2 was observed. Molecular analysis revealed re-expression of tyrosinase-related protein (TRP-1), and tyrosinase in the MIA-deficient cell clones. Both genes are important for melanin synthesis. Transfecting MIA-deficient cell clones with a MIA expression vector revealed downregulation of TRP-1 and tyrosinase expression. It is known that microphthalmia-associated transcription factor MITF, is important for regulating TRP-1 and tyrosinase transcription. Comparing MITF-expression in MIA-deficient cells to wild type melanoma cells revealed equal expression in all cell types. Assuming that downregulation of TRP-1 and tyrosinase might be due to expression of a negative regulator of MITF, we analyzed expression of the recently published MITF-inhibitor PIAS3. PIAS3 (protein inhibitor of activated STAT) interacts directly with MITF and inhibits transcriptional activity of MITF. RT-PCR showed high expression of PIAS3 in the melanoma cells but not in the MIA-deficient cells suggesting an induction of PIAS3 expression in melanoma cells.
In summary, there are phenotypic and morphologic differences in melanoma cells compared to MIA-deficient melanoma cells. In particular the differences in pigmentation will be further investigated.

P176

Efficient cell type-specific induction of apoptosis in melanoma cells by a tyrosinase promoter-CD95/Fas ligand construct requires both tyrosinase expression and CD95/Fas receptor surface expression

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The limited response of malignant melanoma to conventional anti-cancer therapies may partly arise from apoptosis deficiency. We have previously shown expression of CD95/Fas receptor mRNA in 15/17 human melanoma cell lines but complete lack of CD95/Fas ligand. Apoptosis was efficiently triggered in human melanoma cell lines in vitro and in xenotransplants by expression of CD95 ligand. For a possible therapeutic approach based on proapoptotic genes, targeted expression is a prerequisiste. Tyrosinase-derived promoters seem to be suited for melanoma due to the high and restricted expression of tyrosinase in pigment cells. The majority of 10 melanoma cell lines investigated for CD95 surface expression by FACS analysis revealed strong expression, with the exception of Bro and MeWo where expression was at the detection limit. In subsequent studies, a tyrosinase-derived promoter was used for targeting of CD95 ligand expression. Relative activities of the tyrosinase- and a SV40 control promoter were determined by luciferase reporter gene assays in pigmented and amelanotic melanoma cells and in non-melanoma cell lines. In the pigmented melanoma cell lines SK-Mel-13 and MeWo, the tyrosinase promoter was as strong or even stronger as the SV-40 promoter, whereas, it was less than 5% of the SV40 promoter in cell lines A-375 and M-5 characterized by weak or no expression of endogeneous tyrosinase. In the control cell lines, the tyrosinase promoter was generally weak or even at the detection limit. After transfection of a tyrosinase promoter-CD95 ligand construct apoptosis was efficiently induced in the pigmented melanoma lines and also in the almost tyrosinase-negative A-375, which however was characterized by high CD95 surface expression and high sensitivity to agonistic CD95 activation. However, apoptosis induction was only weak in tyrosinase-positive MeWo, which was almost negative for CD95 surface expression. Thus, selective targeting by tyrosinase promoter-CD95 ligand constructs can be applied to melanoma cells positive for tyrosinase as well as for CD95 surface expression.

P177

New cutaneous T-cell lymphoma associated antigens identified by SEREX

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Cutaneous T-cell lymphoma (CTCL) is a heterogeneous neoplasm of the immune system with primary manifestation in the skin. In the advanced stage, CTCL is difficult to treat, thus, immunotherapeutical strategies are developed. As a basis for such therapies we searched for new CTCL-associated antigens using the serological analysis of a recombinant cDNA expression library approach (SEREX). 1.7 Mio recombinants of a cDNA library established from a CTCL cell line (SeAx) were subjected to allogeneic screening with 12 sera from CTCL patients. We could identify 9 positive clones (HD-CL-10 to HD-CL-18) representing 8 different genes, while HD-CL-14 is homologous to chromosomal sequences without a known transcript. HD-CL-13, HD-CL-15 and HD-CL-18 are new genes represented only as ESTs in the databases, whose protein functions have not yet been investigated. The remaining five clones have high homology to previously described genes: HD-CL-10 is similar to the human polypeptide chain Elongation Factor-1 alpha, but lacks the first 251aa. HD-CL-11 is a truncated form of human Prenylcysteine Carboxymethyltransferase without the enzymatic domain. HD-CL-12 is similar to the zinc finger gene 195, but lacks the first 150aa. HD-CL-16 is identical to Cyclophilin A and HD-CL-17 is homologous to the ribosomal protein S28, but shows differences in the protein sequence. Testing sera from CTCL patient (n=12), as well as from healthy controls (n=8), we found that 6 clones were recognized by at least one CTCL serum, but not by any serum of the healthy donors. The mRNA expression was tested for selected antigens by RT-PCR. The Elongation Factor-1 alpha was expressed in 12/15 control tissues, HD-CL-12 in 14/16, HD-CL-13 in 6/18 and HD-CL-15 in 13/16. This contrasted the specificity of the humoral response against these clones, which makes them at least potential targets for diagnostic and prognostic purposes. Further quantitative analysis of their mRNA and protein expression will help to estimate their possible value as targets for immune therapy.

P178

Soluble factors derived from high invasive melanoma cells activate stromal fibroblasts and microvascular endothelial cells

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Invasion and metastasis of human malignant melanoma involves a series of sequential steps including penetration of various connective tissue barriers e.g. basement membranes and the dermal connective tissue. One of the important events involved in tumour growth is the ability of tumour cells to induce vessel formation, a process characterised by intense matrix remodeling achieved by matrix metalloproteinases and serine proteinases. Cell surface receptor-mediated interactions of tumour cells with the surrounding structural and cellular components of the stroma and soluble factors released by the tumour cells are likely to contribute to the activation of stromal cells associated with increased proteolysis of the matrix.

To better understand the molecular mechanisms involved in the invasion and metastasis of human melanoma we have analysed in co-culture systems the influence that melanoma cells have on cells residing the dermal compartment such as fibroblasts and microvascular endothelial cells.

Our analysis shows that cross-talk between high invasive melanoma cells and dermal fibroblasts occurs through soluble factors such as IL1a and bFGF released in high amounts by the tumour cells. In response to these stimuli, dermal skin fibroblasts express and activate different matrix metalloproteinases. On the contrary, two dimensional co-cultures (plastic) of melanoma cells with human dermal microvascular endothelial cells (HDMEC), grown either in direct or indirect contact, failed to show changes in the expression and activation of metalloproteases. Interestingly, when endothelial cells were cultured in three-dimensional fibrin gels, addition of supernatants collected from melanoma cells induced formation of tubular structures similar to those observed upon treatment of endothelial cells with an angiogenic cocktail. These findings indicate that soluble factors derived from melanoma cells mediate induced neovascularisation.

The molecular mechanisms regulating the induced neovascularisation are under investigation. Alltogether, these data clearly demonstrate the importance of the interaction of tumour cells with sourrounding cells residing the tissue as one of the leading events in tumour invasion.

P179

A genetic prime-boost strategy for melanoma vaccination does not eradicate autochthonous melanocytic neoplasms in the skin of cdk4-mutant mice

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Until recently, the development of melanoma vaccines in mice was performed in experimental models involving transplantable melanoma cells. In patients however, melanoma arises autochthonously in the skin and subsequently metastasizes to other organs. In order to more adequately model the expected clinical situation, we set out to evaluate the efficacy of a candidate vaccine approach in mice genetically prone to develop melanoma following carcinogen treatment of the skin. We utilized mice harboring an oncogenic mutation (R24C) in the germline sequence of the cyclin dependent kinase 4 (cdk4), a protein critically involved in cell cycle regulation. A genetic prime-boost strategy stimulated melanoma antigen-specific cellular immune responses in cdk4-mutant mice which were associated with localized autoimmune destruction of melanocytes. However, we did not observe significant destruction of carcinogen-induced melanocytic neoplasms in the skin with this immunization strategy which was optimised for rejection of B16 melanoma cells. We conclude that experimental melanoma vaccines need to be evaluated in novel melanoma models against autochthonously developing melanomas in order to identify strategies which eventually may be of clinical benefit for patients with melanoma.

P180

Triggering of apoptosis in human melanoma cells by proapoptotic Bcl-2-related proteins

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Deficiencies in proapoptotic pathways may be seen as critical events for tumor development and for therapy resistance. The Bcl-2 superfamily of apoptosis-regulating proteins includes both antiapoptotic (e.g. Bcl-2 and Bcl-XL) as well as proapoptotic factors (e.g. Bax, Bcl-XS and Bik/NBK), and consequently, Bcl-2 proteins may evolve as powerful tools to overcome apoptosis resistance in cancer cells. The melanoma cell lines, we investigated, showed significant expression of Bax, as of the antiapoptotic Bcl-2 and Bcl-XL, whereas expression of proapoptotic Bcl-XS and NBK was weak both at the mRNA and protein level. The ratio between pro- and antiapoptotic Bcl-2 proteins turned out as a critical feature for apoptosis sensitivity of melanoma cells as a high Bcl-2 to Bax ratio was characteristic for melanoma cells resistant to agonistic Fas stimulation and to ceramide. In line with this, stable overexpression of Bcl-2 and also of Bcl-XL converted apoptosis-sensitive melanoma cells resistant to proapoptotic signals. On the other hand, melanoma cells turned out as critically sensitive to overexpression of proapoptotic Bcl-2 proteins as shown by transient and stable transfection of Bax, Bcl-XS or Bik/NBK, and the combination with proapoptotic agents like etoposide, pamidronate, and ceramide resulted in additive proapoptotic effects. Also adenoviral expression of NBK triggered apoptosis in all melanoma cell lines, investigated. Induction of apoptosis was clearly evident due to applying tetracycline-regulatable expression systems. In an in vivo model, induced expression of Bcl-XS was further able to significantly reduce the size of xenotransplanted melanomas in nude mice. With respect to the mechanism, by which Bcl-2-related proteins may induce apoptosis in melanoma cells we found both strong DNA fragmentation and nuclear condensation after Bcl-XS induction, whereas several experiments were indicative for an only subordinated role of caspases and cytochrome C release. In summary, these data demonstrate the important role of Bcl-2-related proteins for apoptosis regulation in melanoma cells and may open a series of possible strategies for melanoma therapy.

P181

Simultaneous inhibition of VEGFR1 and VEGFR2 signaling is required for suppression of experimental melanoma metastases

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Formation of blood-borne metastases depends on a series of events, including survival and initial proliferation of cancer cells after extravasation from the capillary bed of secondary organs. Accumulating evidence suggests that the host microenvironment as critical determinant of the proliferative behavior of tumor cells is affected by bone marrow-derived hematopoietic and endothelial stem/progenitor cells. In addition, incorporation of stem/progenitor cells may facilitate angiogenesis-dependent tumor growth. We herein show that experimental lung metastasis formation (B16 melanoma cells) induces proliferation and mobilization of VEGF receptor (VEGFR)-1+ hematopoietic and VEGFR-2+ endothelial progenitor cells. In transplantation studies, in which mice were reconstituted with bone marrow from LacZ-overexpressing animals to trace bone marrow-derived cells in experimental lung metastasis, the majority of incorporated bone marrow-derived cells displayed a monomyelocytic phenotype. In addition, lacZ-positivity was frequently detected in tumor-associated fibrin clots and thrombi, also suggesting mobilization of megakaryocytic cells and platelets from the bone marrow. In approaches to determine the potential significance of VEGFR-1- and VEGFR-2-dependent signaling in blood-borne metastasis formation, the impact of anti-VEGFR antibodies on experimental lung melanoma metastasis was analyzed. These studies showed that both VEGFR-2 and VEGFR-1 signaling were functionally important for hematogenous metastasis, as only simultaneous antibody-mediated inhibition of VEGFR-1 and VEGFR-2 signaling suppressed lung melanoma metastasis formation. Hence, both VEGFR-1 and VEGFR-2 signaling appear to be critically involved in blood-borne melanoma lung metastasis.

P182

VEGF receptors in tumor neovascularization

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Vascular endothelial growth factor (VEGF) and its high affinity receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) are key regulators during embryonic vascular development and tumor angiogenesis. The switch to the angiogenic state in various tumors involves the upregulation of VEGF in tumor cells and the induction of VEGF receptors in the tumor endothelium. Strategies to block VEGF/VEGFR-2 signaling were successfully used to inhibit experimental tumor growth. Here, we analyzed the role of VEGFR-1 in the neovascularization of two different experimental tumors *in vivo*. Retrovirus-mediated gene transfer of VEGFR-1 mutants strongly inhibits the growth of C6 glioma in nude mice and BFS-1 fibrosarcoma in a syngeneic mouse model. Histological analysis of the inhibited fibrosarcoma showed a reduced vascularization, strongly decreased tumor cell proliferation and induction of tumor cell apoptosis. The retroviral gene transfer of the full length VEGFR-1 also strongly inhibited the tumor growth in both models. The BFS-1 fibrosarcoma cells were not infected by the retrovirus, suggesting, that the inhibitory effects in this model were mediated through host tumor endothelial cells. These results underline the central role of the VEGF/VEGFR-1 signaling system in tumor angiogenesis and demonstrate that VEGFR-1 can serve as a target for anti-angiogenic gene therapy.
To increase the specificity of anti-angiogenic gene therapy, it is highly desirable to restrict the expression of therapeutic genes to the tumor endothelium. Therefore, we have identified regulatory sequences of the VEGFR-2 gene, that are sufficient for a strong tumor endothelium-specific reporter gene expression in transgenic mice. These sequences were active in the tumor endothelium of B16 melanoma, BFS-1 fibrosarcoma and oncogene induced adenocarcinoma. The identified promotor/enhancer sequences should therefore represent a valuable tool to target therapeutic genes specifically to the tumor vasculature.

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Dendritic cells infected with MVA-hTyrosinase can induce specific CD4+ T-cell responses *in vitro*

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Since the incidence of invasive malignant melanoma is still increasing new vaccination strategies for tumor prevention and therapy are needed. Recently we could show in a mouse model that tumor-specific IFN-?-producing Th1 cells are highly effective in tumor prevention and therapy of established disseminated lymphoma. In humans the use of gen-transfected dendritic cells (DC) seems to be more effective than the use of peptide pulsed DC for priming naïve T cells. Gene-transfected DC can stimulate T cells independently from HLA-restriction and limited epitope sequences.
Tyrosinase is expressed on more than 90% of malignant melanomas, and is an immunogenic target in autoimmune disease. Therefore we used a modified vaccinia virus ankara encdoing the human tyrosinase gen (MVA-hTyr) for gen-transfer. Monocyte derived DC can be infected with a efficiency of more than 60% and express tyrosinase. Surprisingly, MVA-hTyr infection of DC abolished their capacity to stimulate autologous T cells *in vitro* even in presence of superantigen and IL-2. Phenotype analysis showed a decline of costimulatory molecules and induction of apoptosis in DC. *In vivo* vaccination with MVA leads to a potent immune protection in mice and human. This can be explained by phagocytosis of apoptotic DC by other DC for effective presentation. Thus, coculture of infected DC with non-infected DC restores the stimulatory capacity of DC in superantigen triggered T-cell stimulation. Using MVA-GFP incorporation of apoptotic DC by other DC could be shown. Three rounds of stimulation of autologous $CD4^+$ T-cells by DC-DC-MVA-hTyr led to MVA-hTyr specific $CD4^+$ T-cells as shown by proliferation and cytokine production. Importantly, this *in vitro* generated Th cells presented an IFN-? dominating Th1 cell phenotype.

P184

Contact of High-Invasive but not Low-Invasive Melanoma Cells to Collagen I Induces Increased Secretion of Pro- and Mature Cathepsin B

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The expression of the lysosomal cysteine protease cathepsin B in malignant tumors is highly upregulated and the localization of the protease is altered. In addition to its localization in perinuclear vesicles, previous studies indicate that cathepsin B is also secreted and becomes associated with the cell surface. Tumor cells secrete procathepsin B and both active forms of cathepsin B, but yet the mechanisms underlying these processes are not well understood. Therefore, we investigated to which extent the interaction of tumor cells with the extracellular matrix component, collagen type I, regulates the expression and especially the secretion of cathepsin B. Four melanoma cell lines (MV3, SkMel28, SkMel23, WM164) differing in their invasiveness were cultured for 48h either as monolayers or in 3D collagen I lattices. After a 24h serum-free incubation period the supernatants were analyzed via immunoblotting. Low-invasive cells (SkMel23 and WM164) showed no remarkable amount of secreted cathepsin B, even after they were seeded into collagen I lattices. SkMel28 cells (intermediate-invasive) secreted both active forms of the protease after contact to collagen I. Cultured as monolayers, only the high-invasive MV3 cells constitutively secreted procathepsin B. Due to cell-collagen I interaction the amount of secreted proform was strongly increased and the secretion of both active forms was induced. Furthermore, we could assess the proteolytic activity of secreted cathepsin B by the use of our recently described technique of gelatin zymography under acidic conditions. The zymogramms also demonstrated that in addition to cathepsin B the melanoma cell lines secrete other active lysosomal cysteine proteases, but in smaller amounts when compared to cathepsin B. Based on these results we propose, that secretion of mature cathepsin B is non-selective and a consequence of lysosomal exocytosis induced by the interaction of invasive melanoma cells with collagen I. Increased secretion of procathepsin B is exclusively observed in high-invasive cells and might be due to alterations in normal trafficking pathways enhancing their invasive capacities in the extracellular matrix.

P185

Differential Induction of Connexins 26 and 30 in Skin Tumors and their Adjacent Epidermis

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There is a variety of skin tumors, which can be divided into epithelial and non-epithelial skin tumors. Gap junctions have been shown to play a role in tumor progression. We here show that the synthesis of the gap junction proteins Connexin 26 and 30 (Cx26 and Cx30) is induced in keratinocyte-derived epithelial skin tumors while Cx43 is downregulated. Cx26, Cx30 and Cx43 are absent in non-epithelial skin tumors. Further, Cx26 and Cx30 are induced in the epidermis adjacent to malignant melanoma while they are absent in the epidermis adjacent to benign non-epithelial skin tumors (melanocytic nevus and angioma). The epithelial skin tumors are very heterogeneous concerning the Cx26/Cx30 pattern in the epidermis at the edge of the tumors. We did not observe any difference in the localization pattern of the very similar proteins Cx26 and Cx30 but a variation in intensity of immunoreactivity. As the expression pattern of Cx26 and Cx30 is not identical with CK6, a marker for hyperproliferation, and CK17, a marker for traumatization, we discuss that the induction of these gap junctional proteins is not a reflection of reactive hyperproliferative or traumatized epidermis. We further discuss the putative roles of these gap junctional proteins in tumor progression.

P186

Mutations in the BRAF kinase gene correlate with rapid melanocytic lesion growth

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Mutations in the BRAF-gene have been found in benign and malignant melanocytic lesions and the biological impact of this mutation is still unclear. In cell culture, the $BRAF^{V599E}$ mutation results in a constitutively active kinase function and in increased cell proliferation. We therefore addressed the question whether the occurrence of this mutation in vivo is associated with a rapid growth of melanocytic lesions. Using the digital epiluminescence image archive of the Pigmented Lesion Unit of the department we selected 49 melanocytic lesions, which did not meet the criteria of melanoma at the initial presentation. These lesions were excised 3-4 months later because of an increase in size or a change in structure. For comparison 35 lesions with no clinically visible changes during the same follow up period were randomly selected (which also were excised at the second visit for other reasons). BRAF mutations were identified by PCR followed by sequencing. Among the 35 lesions without changes of size or structure, which were all identified as nevi by histology, a $BRAF^{V599E}$ mutation was found in 2 lesions. Among 13 lesions with structural changes, BRAF mutations were found in 3 melanomas and 1 nevus. Among 36 lesions with an increase in size BRAF mutations were found in 11 melanomas and 5 nevi. In other words it is 7 times more probable for a pigmented lesion with structural changes to have a mutation within the BRAF gene. The odds for a rapid growing pigmented lesion to have the $BRAF^{V599E}$ mutation are 13 times higher than to have the wild-type gene. We conclude that the somatic $BRAF^{V599E}$ mutation is significantly associated with a rapid growth of melanocytic lesions. Mechanisms controlling growth stop in benign lesions despite the presence of the BRAF mutation are under investigation.

P187

Loss of EphB6 expression in metastatic melanoma

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Overexpression of various members of the Eph receptor tyrosine kinases and their ephrin ligands has been frequently reported in cancer. In contrast, a loss of EphB6 gene expression has been correlated with a bad prognosis in human neuroblastoma, suggesting a distinct role of this receptor compared to other family members. More recently, an important role of EphB6 signalling in T-cells has been described, suggesting possibly deleterious immunologic effects of a loss of EphB6 in cancer progression. Therefore, we investigated the expression of EphB6 in melanocytic tumors. EphB6 mRNA of 22 microdissected tissues (7 benign nevi, 7 melanomas, 8 metastases) and 10 different cell lines (normal melanocytes, non-metastatic/metastatic melanoma cell lines) was measured by quantitative Real-Time RT-PCR. For visualization of EphB6 protein expression, immunohistochemistry of 32 melanocytic lesions was performed. On the mRNA level, the benign nevi revealed the highest EphB6 expression (mean=1.43), while melanomas (mean=0.63) and metastases (mean=0.08; p=0.001) displayed a progressive and significant reduction of EphB6 expression. Accordingly, established melanoma cell lines with metastatic potential showed low EphB6 expression in comparison to normal melanocytes and to most of the melanoma cell lines. Immunohistochemistry revealed homogeneous staining in common nevi, whereas in malignant melanomas and metastases a heterogeneously positive to completely negative EphB6 staining was observed. Remarkably, Spitz nevi stain like ordinary melanocytic nevi. Taken together, we show that melanoma progression to metastatic disease is associated with a significant reduction of EphB6 gene expression which may have considerable consequences for the prognosis of malignant melanoma patients and possible gene-therapeutic approaches.

P188

Protein expression of basic fibroblast growth factor (bFGF), FGF receptor-1 (FGFR1), stem cell factor (SCF) and c-kit in melanoma

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The constitutive production of a whole variety of growth factors and cytokines represents a major characteristic of melanoma cells, while normal melanocytes depend on the expression of essential growth factors by keratinocytes and fibroblasts from the immediate environment.
SCF and bFGF are among the most important growth factors for melanocytes that possess the respective receptors, c-kit and FGFR1. Both factors may be involved in melanoma induction as shown previously. While expression of bFGF has been associated with melanoma progression, c-kit has been reported to be downregulated in advanced melanoma. We now seeked to analyze bFGF/FGFR1 and SCF/c-kit co-expression in situ in melanomas by immunohistochemistry. Of 37 melanomas (4 melanoma in situ, 31 primary melanomas, 2 metastases), 81-84 % showed expression of SCF and c-kit, respectively. While c-kit reactivity was most prominent in epidermal and junctional areas and commonly absent in dermal areas, SCF was found in all tumor compartments. Co-expression of SCF (ligand) and c-kit (receptor) was observed in 64 % and was associated with increased tumor thickness indicating an autocrine growth mechanism. bFGF was analyzed in 18 melanomas (2 melanoma in situ, 14 primary melanomas, 2 metastases) of which all showed cytoplasmic bFGF (low molecular weight form of 18 kD) and 37 % nuclear bFGF (high molecular weight forms of 22, 22.5, 24 kD), which has been suggested to act through intracellular pathways independent of cell-surface FGF receptors. FGFR1 was detected in 89 % of all analyzed melanomas.
In summary, our data demonstrate a high frequency of SCF and c-kit protein expression in melanoma tissues, whereby co-expression of both factors in a subset of melanomas may represent an additional autocrine growth mechanism comparable to that of bFGF/FGFR1.

P189

Limits of cell culture: Human melanoma cell lines harbour a murine leukemia virus.

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Stimulated by earlier reports on the presence of retroviruses in mouse and hamster melanoma cell lines, we addressed the question whether human melanoma might be associated with a retrovirus. Testing of supernatants of melanoma cell lines by the product-enhanced reverse transcriptase (PERT) assay showed reverse transcriptase (RT) activity in the human melanoma cell lines SK-MEL-25, SK-MEL-28, MEL-JUSO and MML-I whereas cell lines MeWo, A-375, Colo-38 and BS-780 were negative. The RT activity peaked at a buoyant density in sucrose typical for retroviruses. From this peak fraction a R-U5 sequence indistinguishable from murine leukemia virus (MLV) was identified. Semi-quantitative MLV-specific RNA-PCR demonstrated colocalization of the MLV-like RNA and RT activity on the sucrose gradient of SK-Mel-25. MLV RNA and DNA were also detectable in culture supernatants of SK-MEL-28, MEL-JUSO and MML-I, but not of MeWo, A-375, Colo-38 and BS-780. Sequence comparison revealed highest homology with the RET sequence previously identified in mouse myeloma SP2/0-AG14 cells. Taken together, our data strongly suggest that certain melanoma cell lines are productively infected by a MLV which was probably introduced during tumour passage in mice or by laboratory contamination many years ago and subsequently spread to other lines. Whatever the exact source, investigators working with these cell lines have to be aware of this potential contamination.

P190

A milestone in melanoma genetics: The B-raf oncogene is affected in a substantial subset of melanoma resection specimens.

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In primary melanoma resection specimens, several tumor suppressor genes and oncogenes, among them Ras, are rarely mutated. Downstream of Ras, the serine/threonine kinase *B-raf* has recently been reported to be mutated, among other carcinomas, in a majority of melanoma cell lines with a preponderance of mutations within the kinase domain including the activating V599E transition (Davies et al., Nature 417: 949). We therefore investigated a representative number of melanoma resection specimens for the presence of mutations within the activation segment (exon 15) of the *B-raf* kinase domain. Paraffin embedded primary cutaneous melanomas were microdissected, and DNA isolates were investigated for the presence of *B-raf* exon 15 mutations by polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) gel electrophoresis, followed by DNA cloning and sequencing. Molecular data were correlated with tumor characteristics and with patients' course of the disease. In addition to primary melanomas of the skin, we investigated resections specimens of primary anorectal melanomas and cutaneous/subcutaneous melanoma metastases. Finding mutations in a substantial subset of melanoma resection specimens, *B-raf* alterations possibly affect melanocyte-specific pathways controlling proliferation and differentiation. Inhibition of *B-raf* activity may be a future strategy in the treatment of melanoma.

P191

Molecular changes upon CD26 re-expression in melanoma cells

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CD 26 a type-II membrane protein - also known as dipeptidyl peptidase IV - is a multifunctional enzyme covering functions as costimulatory molecule during T cell activation as well as receptor for extracellular matrix molecules like fibronectin and collagen. It functions as a cell surface peptidase. Among the substrates for the intrinsic serine protease activity there are chemokines like RANTES, MCP 1-3 and SDF1a. CD26 is expressed on epithelial cells of the intestine, on cells of the proximal tubuli of the kidney and on activated T-, B-cells and activated NK cells. It can be detected as a secreted form in the serum.
Van den Oord described that CD26 expression on melanocytic cells is inversely related to the tumor stage of melanocytic lesions (1997). Later, Umadevi et al. demonstrated, that CD26 re-expression suppresses the malignant phenotype in melanocytic cells (1999). To these results Pethiyagoda added further data, that CD26 inhibits the invasive capacity of melanoma cells (2001). But the molecular signalling basics for these features of the molecule were not analysed so far.
To address the question of how CD26 regulates the phenotype of a melanocytic cell, we re-expressed CD26 in a melanoma cell line which does not express CD26 protein neither intracellularily nor on the cell surface. CD26 transfected clones were analysed by FACS analyses for CD26 surface expression. The CD26 expression status of the cell lines was also confirmed by Westernblot and during gene expression profiling. The gene expression profiles was determined by Clontech human 8k plastic arrays. By this approach we could detect the modulation of the expression of multiple genes such as p53, agouti, eucaryotic translation elongation factor 1a and a downregulation of beta-actin and GAPDH expression, which coincides with a slower growth rate. In addition, the CD26 expressing cells exhibited a higher level of attatchment to fibronectin in Boyden chambers. The analysis of the protein tyrosine phosphorylation pattern demonstrated reproducibly a differential phosphorylation of phosphotyrosine proteins of a size of 75 kDa and 42 kDa. The remaining phosphotyrosine pattern was not changed. The identification of these proteins might give rise to the identification of the signaling pathway of CD26 leading to the characteristic malignancy suppressive phenotype.

P192

Mage-3 Specific CD8 Lymphocytes Induced by Vaccination with Peptide Loaded Dendritic Cells Home to Melanoma Metastasis.

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Tumor immunotherapy, especially in melanoma, has gained increasing attention and promising success has been obtained recently. In this study, we investigated tumor specimens and PBMC from 5 patients with stage IV melanoma prior and following an immune vaccination therapy with Mage-3 loaded dendritic cells (DC). Beside one patient, all experienced a partial but objective tumor regression. A significant increase of intra-tumoral Mage-3 specific CD8 T cells following DC vaccination was found by in-situ Mage-3/HLA-A1 multimer staining. These data correlate with the detection of Mage-3 specific CD8 cells following vaccination in the peripheral blood as documented by tetramer-FACS. The ELISPOT from PBMC revealed a IFN-gamma secretion after stimulation with Mage-3 in 4 patients. By comparative clonotype mapping, an oligoclonal T cell response infiltrating the tumor lesions was found in all 5 patients. Notably, the T cell clones detected in the tumor specimens were in part identical to those found in the patients' PBMC. The number of expanded T cell clones in the inflammatory infiltrate of tumor lesions prior and after vaccination increased following DC vaccination and, as analysed quantitatively by real-time PCR, signals for selected clonal responses were significantly higher. Taken together, this analyses documents that: (1) Ag-specific CD8 T cells are inducible by DC vaccination loaded with Mage-3; (2) Mage-3 specific CD8 cells are present in the tumors; (3) identical T cell clones can be detected in the peripheral blood and in the tumor lesions, and (4) DC vaccination seems to boost Mage-3 specific T cell responses. Further studies are now required to analyze the phenotype and functional status of these Mage-3 specific CD8 T cells.

P193

Identification of differentially expressed genes in metastatic melanoma compared to primary melanoma

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In order to develop effective treatment strategies for patients with advanced melanoma it is important to gain a better understanding of the underlying mechanisms of melanoma development and progression. The main objective of our work is to find molecular targets for the effective treatment of advanced melanoma. For the identification of differentially expressed genes in metastatic melanoma compared to primary melanoma we first isolated pure tumour cells from frozen tissues by laser capture microdissection (LCM). Subsequently, the RNA was reverse transcribed and the cDNA amplified by SMART (Clontech). Afterwards, PCR-amplified subtractive amplification was used to enrich differentially expressed genes in primary melanoma or lymph node metastasis. In total, 320 clones from each subtraction was screened by dot blot. Positive clones were sequenced and screened by Northern blot and in situ hybridization. The number of differentially expressed genes isolated from metastatic tumour cells was higher than the number of genes isolated from primary tumour cells indicating that during progression several genes are activated. Beside ribosomal and mitochondrial genes several genes involved in cell proliferation and transcriptional regulation were found to be differentially expressed in metastatic melanoma cells. Interestingly, several genes with unknown function which have never been associated with melanoma development were also isolated. Analysis of expression and function of these genes will reveal whether they play a role in melanoma progression.

P194

Expression analysis of the transcription factor YB-1 in malignant melanoma

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Using PCR-amplified subtractive hybridization of melanocytic nevi and primary melanoma tissues we isolated the DNA-binding protein dbpB/YB-1 overexpressed in primary melanoma. YB-1 is a transcription factor regulating the expression of several genes involved in tumour development and progression. The role of YB-1 in melanoma development is unknown. To analyse YB-1 protein expression in melanoma progression we generated a polyclonal antiserum against YB-1 and performed semiquantitative western blot analysis and confocal laser scan microscopy of melanoma cell lines and tissues of melanocytic nevi, primary melanoma and melanoma metastases. The expression analysis of melanocytes and melanoma cell lines which are at different stages of tumour progression indicated that in the cause of melanoma progression a translocation of YB-1 from cytoplasm to the nucleus takes place. Melanocytes and radial growth phase (RGP) melanomas showed only a cytoplasmic YB-1 protein expression whereas in vertical growth phase (VGP) melanomas and metastatic melanomas YB-1 is expressed in most cases in the cytoplasm and in the nucleus. In tissues from primary and metastatic melanoma we observed an increased YB-1 protein expression level and a translocation to the nucleus. These results indicate that during melanoma progression YB-1 protein is translocated to the nucleus. Functional assays should indicate whether the transcription factor YB-1 plays a role in invasion and metastasis of melanoma.

P195

Bcl-2 antisense oligonucleotide treatement sensitizes ceramide-resistant melanoma cells to apoptosis

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Malignant melanoma is one the most aggressive tumors described to date. As most antitumor strategies result in tumor cell apoptosis, regulation of apoptosis represents an important mechanism by which melanoma cells may escape from therapeutic treatment. Cell sensitivity to programmed cell death is strongly modulated by members of the Bcl-2 family, as the balance of homo- and heterodimer formation between pro- and anti-apoptotic members of this family may define apoptosis susceptibility in the cellular context. As we have shown previously, expression of Bcl-2-related proteins strongly varies among different melanoma cell lines, and the ratio of pro- and antiapoptotic proteins may be decisive for CD95/Fas- and for ceramide-resistance or sensitivity (Raisova et al., J. Invest. Dermatol., 2001, 117:333-40). Based on these results prosurvival Bcl-2-related proteins are attractive intracellular targets to sensitize melanoma cells to apoptotic cell death. Antisense oligonucleotides targeting Bcl-2 have been shown to be useful tools to facilitate apoptosis in various tumor types. In malignant melanoma the bcl-2 antisense oligonucleotide G3139 (Genasense ™|) has been shown to be a potent inducer of apoptosis. To further clarify the role of Bcl-2 as prosurvival factor we investigated the effect of Bcl-2 phosphorothioate oligonucleotides in three ceramide-resistant human melanoma cell lines. After treatment with antisense oligonucleotides, no significant changes at mRNA level were detected. By Western blot analysis we found a significantly reduced Bcl-2 protein expression after antisense treatment in all cell lines investigated, which varied between 40% and 80% as compared to cells treated with control oligonucleotides. In strong correlation, apoptosis was inducible by ceramide in the former resistant cells after treatment with Bcl-2 antisense oligonucleotides. These results underline the pro-apoptotic effect of Bcl-2 antisense strategies in melanoma and further suggest a central role of ceramide in mediating pro-apoptotic signals.

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Melanotransferrin - An known melanoma marker with new perspectives

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Malignant melanoma is a highly invasive and metastatic tumor with increasing incidence and mortality. As adjuvant therapy of proven efficacy is not currently available, the search for specific markers for those tumor subtypes most likely to metastasize may lead to the development of new prognostic indicators. Several factors that predict the survival of the patients have been described. To date, most studies have shown that the best predictive and sensitive marker for the outcome is the depth of tumor invasion (Breslow thickness). However, additional variables are needed with improved value for melanoma patients. Thus, a tremendous number of melanoma associated antigens have been characterized to provide insight into the prognosis of melanoma progression.

Therefore, mice were immunized with sublethal irradiated melanoma cells. A panel of monoclonal antibodies recognizing melanoma cells was generated. Here, we report one of them, the monoclonal antibody B-E11, which recognizes melanoma cells in tissue sections depending on the level of the invasion depth (Breslow thickness). The mAb B-E11 does not stain melanoma cells in melanomas with < 1,5 mm invasion depth. In contrast, melanomas invading the dermis for more than 1,5 mm were all positive for B-E11. These results are of clinical significance, because melanomas with a tumor thickness of > 0,75 mm were shown to have a more worse prognosis. Furthermore, B-E11 strongly stains all melanoma metastases. In vitro, mAb B-E11 recognises 12 of 17 melanoma cell lines derived from melanoma and melanoma metastases. However, there are considerable differences in the binding of the antibody to the melanoma cell lines (30-90%).

To elucidate the biochemical nature of the protein recognised by the mAb B-E11 the protein was isolated using the mAb BE11 and magnetic beads. The corresponding antigen was shown to be the known melanoma molecule p97 also called melanotransferrin because of its high expression on melanoma cells and its homology to the iron binding protein transferrin.

Until now, melanotransferrin is described as an universally expressed protein on melanoma cells. However, our data show, that melanotransferrin is an interesting molecule in respect to melanoma progression.

P197

Discriminating between benign and malign melanocytes: Is the splicing factor SF2/ASF helpful to achieve this goal?

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The reliable discrimination between benign and malign melanocytes is a major unsolved problem in histopathology of the skin. Seeking for reliable markers of melanoma cells we developed monoclonal antibodies against a mixture of available melanoma cell lines. One of the monoclonal antibodies (moAb BA-8) specifically stained all melanomas investigated by immunohistochemistry. In cell-rich tumours preferentially melanoma cells at the tumor-stroma-border were detected whereas metastases are poorly stained. In contrast, naevus cells in the epidermis as well as in the dermis of benign melanocytic lesions were never stained by this antibody. Healthy skin was mostly free of signal with the exception of some keratinocytes of the outer root sheath of the hair follicle.
Using affinity chromatography with the coupled moAb BA-8 the corresponding antigen was purified. MALDI-TOF analysis revealed that this IgM-antibody specifically binds the serine-arginine protein SF2/ASF, a transacting splicing regulator that is important in regulating the expression of specific protein isoforms derived from alternative splicing of single pre-mRNA (e.g.: apolipoprotein B). Further SF2/ASF can mediate topoisomerase activities finally changing the degree of DNA relaxation.
Together with data from other groups our data indicate, that SF2/ASF-expression might be associated with cell transformation during tumor formation and therefore might be of importance for the development of primary melanomas.

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Virus-Like Particles of HPV92, a new type isolated from a Basal Cell Carcinoma

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Infection with human papillomaviruses (HPV) induce usually benign papillomas (warts) of skin and mucosae. High-risk HPV16 and 18 are the major cause for cervical carcinoma whereas HPV5, -8 and related types induce skin cancer in sun-exposed areas of patients with the rare genodermatosis epidermodysplasia verruciformis and organ transplant recipients. However, an etiologic role for HPV in the development of non-melanoma skin cancer (NMSC) in immunocompetent individuals is still unclear. Recently, a novel HPV type, designated (candidate) HPV92, has been isolated from a basal cell carcinoma of an immunocompetent Australian male. HPV92 is phylogenetically distantly related to HPV5 and -8. Using recombinant baculoviruses, L1 major capsid proteins of HPV92, 5 and 8 were expressed in Sf9 insect cells and purified by density gradients. Electron micrographs (negative stain) demonstrated spherical structures of approximately 50 nm diameter indicating that L1 of HPV92 self-assembled into capsomeric virus-like particles (VLP) similar to other types. Immunization of a NZW rabbit (4x 50µg HPV92 VLP in Freunds adjuvant) elicited high titer antibodies (>10.000) to L1. By Western blotting and VLP-ELISA, HPV92 immune sera cross-reacted with L1 of HPV5 and -8. We next examined sera by hemagglutination inhibition (HAI) assay as surrogate for viral neutralzing activity. In contrast to preimmune serum, HPV92 immune sera scored positive using homologous HPV92 VLP, but not HPV5 or 8. In contrast, an HPV5 immune serum had HAI activity to all three VLP types indicating that HPV5 VLP display immunogenic cross-reacting epitopes. Pooled sera of 10 patients each with squamous or basal cell carcinoma displayed HAI activity against HPV92, 5 and 8 VLP. In contrast, sera from normal volunteers inhibited hemagglutination by HPV92 only indicating common exposure in the normal population. Larger seroepidemiologic studies including patients with a variety of epithelial proliferations will provide information on a possible role of specific HPV types in the pathogenesis of NMSC. VLP of HPV92 or additional high-risk types are potential candidates for a prophylactic vaccine for patients at skin cancer risk.

P199

Ephrin-B2 dependent cell-cell contact signaling induces migration competence and apoptosis resistance in B16 melanoma cells

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Ephrins are well characterized cell-surface bound cell-cell signaling molecules that represent key regulators of cell migration in organogenesis. Since they are frequently overexpressed in various cancers, a functional role in cancer progression has been postulated, but the evidence is still scarce. Based on our findings that Ephrin-B2 is upregulated in the invasive front of advanced human malignant melanomas (MM) and metastases, we addressed the question, how this pathway may contribute to MM progression. Therefore, we studied the effects of both constitutive overexpression by transfection of B16 cells with the complete or a dominant-negative, signaling-deficient construct, and the transient activation by treatment with recombinant Ephrin-B2 activating fusion-proteins: Upon activation of this pathway the cells adopt a highly migration-competent phenotype characterized by induction of multiple lamellipodia, a high rate of actin polymerisation, activation of focal adhesion kinase (FAK), and integrin-receptors. Functionally, this confers a significant increase of attachment of the cells to fibronectin coated surfaces. Furthermore, the migratory net effect is a significant increase of migration/invasion in both scratch wound healing assays and in Boyden chambers. Apart from that, we show that due to activation of MAP kinases (JNK), there are also multiple changes occurring on the transcriptional level. Affymetrix® chip analyses revealed that Ephrin-B signaling is linked to upregulation of 14-3-3 zeta, a co-activator of the B-Raf pathway, and several other factors (SOX-4, ATF-2), which have been linked to the vertical growth phase and the metastatic phenotype. Consequently, in C57Bl6-mice, subcutaneous MM expansion was significantly reduced, if dominant-negative transfected cells were injected compared to mock-transfected ones. Accordingly, the rate of apoptotic, TUNEL-positive cells was grossly enhanced. We conclude, that cell-cell contact signals via the Ephrin-B pathway can modulate migration and invasion of MM cells. Further links to other cancer-pathways (B-Raf, apoptosis) make it a candidate for molecular targeted future therapies.

P200

Identification of signature genes for leukemic CTCL cells in S?ry syndrome

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S?ry syndrome (SS) is a leukemic variant of cutaneous T cell lymphoma (CTCL) of unknown etiology. The occurrence of leukemic $CD4^+$ T-cells is necessary to define the syndrome; however, specific diagnostic markers are missing. In an attempt to identify singly informative traits with potential diagnostic applicability, we performed global gene expression profiling on peripheral blood mononuclear cells from classical SS patients as compared to healthy donors (CTR). Using Affymetrix DNA Chip analysis, 82 genes were found to be differentially expressed with a fold change of > 2 in every SS patient/CTR pair analysed. Out of these, 19 genes were over-expressed in SS patients including 4 genes with unknown function named SS-associated molecules (SSAM)1-4, genes involved in cell signalling (TOX, TRIM), immune responses (CD28), and cell growth / death (RGC32, B56, Twist, SIAT8, TFRC). Using quantitative real-time PCR analysis, we confirmed the profiling results for 14 genes and demonstrated up-regulation in disease-related $CD4^+$, but not $CD4^-$ T cells from SS patients. ROC statistical analysis of the data showed that SSAM1-4 and TOX provided perfect discrimination between CTCL and benign inflammatory skin diseases/healthy donors, whereas SSAM4 and TOX were also able to discriminate between SS and MF patients within the CTCL group.

P201

The physical mechanism of pericellular proteolysis in tumor cell migration: the belt-cleavage concept.

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Tumor cell migration through three-dimensional (3D) extracellular matrix involves adhesive and proteolytic cell-matrix interactions. It is not clear, however, where and how the cleavage of ECM fibers occurs along the moving cell?s advancing length axis without challenging adhesive and migratory force generation towards the substrate. Using a 3D fibrillar collagen model, we visualized proteolytic fiber cleavage during migration of HT-1080 fibrosarcoma cells overexpressing MT1-MMP by combining immunological, biochemical and physical analysis using confocal microscopy up to a spatial resolution of 200 nm. Major degradation was visualized at different positions along the length axis, while only minor collagenolysis occurred at the very tip of the leading pseudopod (invadopod). During forward movement, cell volume was gradually gained up to the maximum cell diameter by contact-mediated sequential degradation of only those fibers that had generated belt-like constrictions towards the cell body. At constriction sites, beta 1 integrins and F-actin were strongly colocalized with degradation zones, indicating cooperation of adhesive and proteolytic events. Cleavage dynamics, as visualized by real-time imaging of FITC-collagen fibers, showed that after focalized cleavage collagen fibers were topographically displaced towards parallel alignment along the advancing length axis, resulting in a trail-like matrix defects.This pericellular collagenolysis was almost completely abrogated by broad-spectrum protease inhibitors and, in part, by an antibody blocking beta 1 integrin-mediated adhesion. In conclusion, multidimensional microscopy reveals pericellular proteolysis as a tightly controlled process of sequential focal degradation of individually constraining collagen fibrils, allowing invasive tumor cells to precisely tailor ECM barriers according to their biophysical needs.

P202

Expression of the DNA-mismatch repair enzyme hMSH2 in acquired melanocytic nevi, malignant melanoma and metastases of malignant melanoma and implications for its contribution to the repair of UV-induced DNA damage in melanocytes

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Microsatellite instability (MSI) secondary to replication errors is a genetic mechanism important in the development of various human cancers. This mechanism is characterized by length changes at repetitive loci scattered throughout the genome that can be detected in various malignant skin tumors. It was recently shown that muations in the gene of the DNA repair enzyme hMSH2 are causative for the generation of microsatellite instability and carcinogenesis in hereditary nonpolyposis colorectal cancer. Additionally, it was shown that the hMSH2 gene is regulated by the p53 tumor suppressor protein. Significance of mismatch repair pathways for the repair of UV-light induced DNA damage was recently shown by demonstrating specific binding of human hMSH2/hMSH6 heterodimers to DNA containing UV-light photoproducts. Furthermore, human cells with mutations in particular mismatch repair were likewise found to have a deficiency in transcription coupled repair of UV-induced pyrimidine dimers. We have now analyzed the immunoreactivity of hMSH-2 (mab FE11) and p53 (mab DO-7) in malignant melanomas (MM, n=15), metastases of malignant melanomas (MMM, n=17), and acquired melanocytic nevi (MN, n=8). Nuclear immunoreactivity for hMSH2 was detected in all samples analyzed. Interestingly, semiquantitative analysis revealed increased levels of p53 and hMSH2 proteins in MM and MMM as compared to MN. Expression of hMSH2 and p53 proteins correlated in a proliferation-independent manner (Ki-67). Additionally, we have analyzed the expression of hMSH2 and p53 in epithelial tumor cells (SCL-1) and in melanoma cell line SkMel28 after UV-B irradiation. We found UV-induced upregulation of hMSH2 and p53 mRNA in SCL-1 but not SkMel28 cells. Our findings indicate that (a) inactivating mutations may not be involved in MSI in MM and MMM, as the immunohistochemical staining with FE11 depends on functionally intact hMSH2 protein (b) upregulation of hMSH2 in MM and MMM may be induced by an increase in functionally active p53 protein (c) upregulation of hMSH2 gene expression by the p53 protein may be of importance for the repair of UV-B-induced DNA damage in skin cells.

P203

CD117(c-kit)-independent increase of mast cells at sites of skin carcinogenesis

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We and others have previously shown that mast cells (MC) can modulate the development and growth of skin tumors and that MC numbers are markedly increased at sites of skin carcinogenesis. CD117, the receptor for stem cell factor (SCF), is crucial for MC proliferation and chemotaxis, both of which are possible mechanisms for MC accumulation at sites of skin tumor formation. Here, we have used genetically CD117(c-kit)-deficient Kit^{W}/Kit^{W-v}-mice to assess the role of SCF in MC hyperplasia at tumor sites. 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 15 weeks), which resulted in the induction of skin papillomas (after 9 weeks) and subsequent malignant conversion (after 25 weeks of treatment). Most interestingly, MC numbers were found to be dramatically increased in tumor affected skin in the absence of functional CD117 expression. MC numbers in CD117(c-kit)-deficient mice were increased 125-fold and 260-fold at week 15 and 25 of treatment, respectively. In contrast, normal *Kit*+/+-mice only showed a 6-fold increase of MC numbers at these time points (Kit^{W}/Kit^{W-v}-mice baseline: 0.1±0.1 MC, week 15: 12.5±6.4, week 25: 26.0±7.0; *Kit*+/+-mice baseline 6.5±0.6 MC, week 15: 26.9±1.7, week 25: 31.0±2.7). Whereas in *Kit*+/+-mice increased MC numbers were observed in all areas of DMBA/TPA-treated skin, CD117(c-kit)-deficient mice, surprisingly, developed MC accumulation only at sites of tumor formation. These findings suggest that DMBA/TPA-skin treatment results in SCF-mediated MC hyperplasia, whereas MC accumulation in the vicinity of skin tumors induced by this treatment is independent of c-kit-signaling. Our data provide proof of concept that factors other than SCF promote significant and biologically relevant MC chemotaxis and/or local MC proliferation *in vivo*.

P204

Modulation of phosphorylated histon H2AX (?H2AX) foci by UV- and X-irradiation in hMSH2 -/- and wild type cells

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Skin tumors are frequently a result of UV-induced DNA damage. The hMSH2 gene plays a central role in DNA mismatch repair. Its promoter has been shown to be upregulated in various cell types in response to UV irradiation due to specific binding of a p53/cJun heterodimer. Significance of mismatch repair pathways for the repair of UV-light induced DNA damage was confirmed by demonstrating specific binding of human hMSH2/hMSH6 complexes to DNA incorporating thymine or uracil containing UV-light photoproducts. Furthermore, human cells with mutations in particular mismatch repair genes were likewise found to have a deficiency in transcription coupled repair of UV-induced pyrimidine dimers. Of all the forms of DNA damage, double strand breaks (DSBs) are potentially the most problematic, since they may lead to broken or rearranged chromosomes, cell death or cancer. Increase in phosphorylated histon H2AX (?H2AX) foci have been shown to signal the presence of DNA damage, in particular double strand breaks. hMSH2 deficient tumor cell lines lost most of their ability to accurately repair plasmid DNA double-strand breaks by homologous recombination. UV-light does not directly produce DNA double-strand breaks but rather produces pyrimidin dimers and other photoproducts, that must be removed or bypassed to prevent arrest of the replication fork. UV induced replication arrest in the Xeroderma pigmentosum variant (XPV) but not in normal cells leads to an accumulation of phosphorylated histon H2AX (?H2AX). We have now analyzed effects of UV- and X-irradiation on expression of phosphorylated ?H2AX in hMSH2-defective (hMSH2-/-) and hMSH2 wild type cells using a specific mAB directed against ?H2AX (Trevigen) and immunocytochemical and flow cytometry. Our results indicate that hMSH2 status modulates the level of ?H2AX in response to irradiation, most pronounced to X-irradiation.

P205

Direct 3D observation of GFP-actin in migrating B16-Melanoma cells: dynamic, plasticity and adaptation along on the extracellular matrix structures

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The dynamics of the actin cytoskeleton in response to extracellular matrix structures is the prerequisite for cell polarisation, shape change, and migration, including the invasion and metastasis of tumor cells. In invasive B16-mouse melanoma cells expressing GFP-actin fusion protein we directly imaged cytoskeletal dynamics and movement in response to physically different collagen substrata using time-lapse videomicroscopy and confocal microscopy: 1) cells on 2D surfaces coated with monomeric collagen, 2) 2D surfaces composed of fibrilliar collagen, and 3) cells which were embedded in 3D collagen matrices. On 2D monomeric collagen quick cell adhesion, spreading, and cell flattening were followed by migration driven by focal contacts in which cable like actin structures (stress fibres) inserted. In 3D collagen matrices however, cells developed a spindle like (mesenchymal) shape with cylindrical finger-like pseudopods which generated the forward-driving force towards collagen fibres. These pseudopods contained dynamic polymerized actin yet lacked stress fibres. A similar mesenchymal cell shape and structure of the actin cytosceleton that lacked stringent focal contacts and stress fibres developed on 2D fibrilliar collagen matrices. The migration efficiency in 3D collagen was significantly lower, compared to 2D substrata, suggesting an impact of matrix barriers on the migration velocity. Both, actin polymerization and migration were severely impaired by inhibitors of the actin cytoskeleton (Cytochalasin D, Latrunculin B, Jasplakinolide), causing cell rounding and oscillatory “running on the spot”.
These findings show the dynamics of the actin cytoskeleton in living melanoma cells critically dependent on and respond to the physical structure of the ECM. 3D collagen matrices hence favor in vivo-like cell shape and cytoskeletal organization while flat cell spreading and formation of stress fibres are specific cell characteristics of cells on 2D.

P206

Identification of immunogenic epitopes from the clonotypic T cell receptor and tumor antigens in CTCL

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In order to develop specific cancer vaccines for immunotherapy of CTCL, we identified potential immunogenic epitopes from the T cell receptor (TCR) and from tumor antigens as target proteins by the "reverse immunology" approach. The TCR beta-chain V regions expressed in the malignant T cell clones of two Sezary syndrome patients were sequenced and potential peptides fitting the patient's HLA class I molecules (HLA-A2 and -A3, respectively) were predicted. In a T2 stabilization assay for HLA-A2 peptides, 3 of 5 selected peptides showed intermediate to high binding affinity and 2 of these peptides could induce peptide-specific T cells from peripheral blood mononuclear cells (PBMC) of healthy donors as analyzed by IFN-? secretion (ELISPOT). The peptides from the TCR of the second patient were tested for binding activities by T2 cells transfected with HLA-A3 (T2-A3 cells). One of 3 predicted HLA-A3 restricted peptides with high binding affinity was able to induce IFN-? secretion when stimulated with corresponding CTCL cells in a HLA-A3 restricted fashion. Furthermore, we identified HLA-A3-dependent peptides derived from cTAGE-1 and cTAGE-5a, two newly described CTCL-specific antigens. Immunogenicity of these peptides was tested in vitro by priming of specific T cells using autologous dendritic cells from healthy volunteers. Two cTAGE-5a peptides, as well as one peptide from cTAGE-1 were shown to induce specific T cells in the context of the HLA-A3 allele. As measured by IFN-? secretion in an ELISPOT assay, the reactive T cell lines were capable of recognizing the corresponding peptides pulsed onto a K562-A3 cell line and the tumor cell line SeAx, which expresses HLA-A3 and the tumor antigens. In conclusion, we could identify immunogenic TCR-derived specific epitopes, which appeared likely to induce cytotoxic immune responses in CTCL in the context of HLA-A2 and -A3, as well as several HLA-A3-dependent epitopes derived from tumor-specific members of the cTAGE-family. These results are important for the development of a CTL-based immunotherapy for CTCL and other malignancies.

P207

Autoimmune cutaneous hypopigmentation following DNA vaccination with TRP-2 in SCF transgenic mice - mimicking vitiligo in humans

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The interaction of stem cell factor (SCF) with its receptor, c-kit, is important for migration, differentiation, and survival of melanocytes in the skin. Human epidermal keratinocytes produce SCF, but in murine epidermis the SCF gene is not expressed. The difference in SCF expression between human and mice is reflected by the difference in melanocyte distribution in these two species. In human skin, melanocytes are present in the epidermis. In contrast, melanocytes in murine skin are generally confined to hair follicles that determine coat color in mice.
In our study we used SCF transgenic (tg) mice that express membrane-bound SCF in epidermal keratinocytes controlled by the cytokeratin 14 promoter (Kunisada et al. 1998 J.Exp.Med.187:1565ff). Keratinocyte expression of SCF results in postnatal maintenance of epidermal melanocytes causing epidermal hyperpigmentation throughout the entire life. Thus, SCF tg mice have *pigmented skin* with increased numbers of melanocytes in the epidermis *like humans*.
As shown previously, xenogeneic DNA immunization of mice against melanocyte differentiation antigen Tyrosinase-related protein-2 (TRP-2) could induce tumor immunity and autoimmunity manifested as depigmentation of regrowing hairs. Here, we immunized SCF tg mice against huTRP-2, using the same method of biolistic DNA immunization. DNA encoding huTRP-2 was delivered by particle bombardment into abdominal skin of mice. 8/13 SCF tg mice developed hypopigmentation characterized by white spots at the inoculation site. 10/10 C57/BL6 control mice developed depigmentation of regrowing hairs. No changes in pigmentation or coat color occurred after immunization with a control Null-vector.
Cutaneous melanocytes in most mouse strains are restricted to hair follicles, but in our studies of SCF tg mice that have epidermal melanocytes, which more directly reflects the physiology of melanocytes in human skin, autoimmune cutaneous hypopigmentation has both clinical and histologic characteristics that directly mimic vitiligo in humans. SCF tg mice will be usefull in studies of melanocyte biology and for induction of spontaneus melanoma.

P208

Aberrations of chromosome 7 and 8 in cutaneous T-cell lymphomas analysed by fluorescence in-situ hybridisation

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Cutaneous T-cell lymphomas (CTLC) are associated with a characteristic pattern of chromosomal aberrations (6q-7-8q-13q). In the present study, chromosomes 7 and 8 were screened for numerical and structural aberrations in 19 patients with CTLC using fluorescence in-situ hybridisation (FISH). Centromeric probes were used for chromosome 7 and 8. For the analysis of candidate tumor genes probes for the regions 7q34-35 (encoding the T-cell receptor beta region) and 8q24 (encoding the myc-oncogen) were chosen. Minimum and maximum signals of 2-4% of labeled nuclei were regarded as a cut of levels for the probes, respectively. For chromosome 7 centromeric probes, aberrations were observed in 8 of 19 patients, for chromosome 8 in 9 out of 19 patients. The patients identified by CGH were confirmed by FISH and, moreover, additional patients were identified. 6 out of 10 patients revealed aberrations of the region 7q34-35, and 4 out 10 patients for 8q24. Sequential analysis of tumor samples obtained from patients during the course of the disease revealed a consistent occurence of the aberrations. The results of this study demonstrate that FISH analysis of tumor samples of CTLC is a reliable and reproducable technique. The prognostic relevance of frequent aberrations remains to be determined.

P209

Convergence of the p38/ERK/MSK1 and PKA pathways to CREB phosphorylation for the induction of *c-fos* gene expression by Interleukin-1ß.

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Interleukin-1ß (IL-1ß) plays a central role in acute and chronic inflammation. At the transcriptional level, IL-1ß promotes expression of a variety of immediate-early-response genes (IEGs). In turn, IEGs influence the expression of secondary response genes contributing to the phenotypic response of the cell to proinflammatory stimuli like IL-1ß. It is known that stimulus-dependent gene transcription of the prototypical IEG, *c-fos*, is mediated via several cis-acting elements, including at least three cAMP response elements and a serum response element. However, the precise mechanisms by which IL-1ß induce *c-fos* gene expression remain unclear. Here we show that inhibition of either ERK or p38 mitogen-activated protein kinases (MAPK) abolished IL-1ß-induced *c-fos* gene expression in human HaCaT keratinocytes at the transcriptional level. Our results also indicate that activation of mitogen- and stress-activated protein kinase 1 (MSK1), and subsequent phosphorylation of cAMP response element-binding protein (CREB) following ERK and p38 MAPK activation, were crucial to IL-1ß-induced *c-fos* gene expression. In addition, indomethacin, an inhibitor of cyclo-oxygenase-1/2, and H-89, an inhibitor of cAMP-dependent protein kinase A (PKA), partially abolished IL-1ß-induced CREB phophorylation and *c-fos* gene expression. These results demonstrate that IL-1ß-induced prostaglandin secretion and subsequent PKA activation contribute, together with the p38/ERK/MSK1 signaling cascade, to phosphorylate CREB, hence activate *c-fos* transcription. Taken together, these studies provide additional insights into the molecular mechanisms of CREB-dependent IL-1ß-induced *c-fos* gene expression.

P210

Dissection of proinflammatory intracellular signalling pathways and identification of novel TNF-a-induced target genes in primary human endothelial cells by transcriptional profiling

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Activation of endothelium is pivotal for the recruitment of leukocytes to sites of inflammatory challenge. This process requires tight regulatory control mechanisms which co-ordinately elicit distinct gene expression programs. We here assessed the relative contribution of different intracellular signalling pathways to TNF-a-induced gene expression in primary human endothelial cells after blockade of the NF-?B pathway by stable expression of a dominant negative mutant of the NF-?B upstream kinase IKK2 or after inhibition of the p38 MAP kinase pathway. Thereafter, we defined the TNF-a-induced gene expression profile of endothelial cells by oligonucleotide microarray analysis covering >13,000 genes. Data were validated by extensive statistical analysis and confirmed by real-time PCR and flow-cytometry. The results obtained in this study were threefold: First, we here provide a systematic and statistically validated analysis of the TNF-a-induced gene expression program in endothelial cells. Second, regulation of individual genes was assigned to distinct intracellular signalling pathways. To our surprise, virtually all TNF-a-regulated genes appeared to be controlled by the IKK2/I?Ba/NF-?B pathway when studying cells with impaired NF-?B signalling. In contrast, the expression of a lower number of genes was additionally modulated by the p38 MAP kinase pathway. Third, we also identified novel target genes which are up-regulated or suppressed upon exposure to TNF-a in an IKK2/I?Ba/NF-?B- and/or p38-dependent manner. Our results provide a list of potential candidate genes for targeted modulation of endothelial functions during inflammation.

P211

Hypoosmotic Stress Induces E-Cadherin Expression in cultured Human Keratinocytes

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Human epidermis marks the interface between internal and external environment with the major task to maintain body hydration. Exposure of skin to different environmental conditions, such as drought or bathing in anisosmolar fluids, could perturb the osmotic balance. Particularly, bathing in tapwater represents a hypoosmotic stress that human skin is routinely challenged. The present in vitro approach studied the effect of hypoosmolarity on cell-cell contacts. It was demonstrated that hypoosmotic stress applied to human epithelial cells (HaCaT, A-431) induced upregulation of E-cadherin at both, the protein and mRNA level. Deletion mutants of the E-cadherin promoter identified an element ranging from –53 to +31 that conveyed positive transactivation under hypoosmotic stress. In order to dissect relevant upstream regulators members of the MAP kinase family, the EGF-R and PKB/Akt were investigated. Hypoosmotic conditions led to a fast activation of ERK1/2, SAPK/JNK, p38, EGF-R and PKB/Akt with distinct activation patterns. Experiments using specific inhibitors identified p38 as a regulator of E-cadherin transactivation under hypoosmotic conditions. Finally, these studies provide evidence that E-cadherin is an osmo-sensitive gene that responds to hypoosmotic stress. The function of this regulation may be found in morphological changes induced by cell swelling. It is likely that induction of E-cadherin contributes to the stabilization between adjacent cells in order to withstand the physical forces induced by hypoosmolarity.

P212

Mechanical Pressure-Induced p38 Phosphorylation is Mediated by Protein Kinase C in Epithelial Cells

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Cells within human skin are permanently targeted by mechanical forces of different qualities (e.g. stretching and pressure). In contrast to stretching mechanical pressure is thought to trigger cellular differentiation processes. The underlying molecular signalling pathways which are involved in transduction of mechanical pressure are still enigmatic.
In the present in vitro attempt epithelial cells (HaCaT, Cos 7 and HEK 293-T) were mechanically stimulated by teflon weights that were placed into the culture dishes (1.02 g/cm^2). Cells were analysed after different time intervals using SDS-PAGE and Western blotting. Mechanical pressure applied for a maximum time of 20 min showed a peak phosphorylation for the stress-kinases p38 and c-jun NH_2-terminal kinase-2 (JNK2) between 5 and 10 min. In order to further dissect the signalling cascade, upstream and downstream regulators of p38 were examined. Downstream of p38 the phosphorylation of the small heat shock protein 27 (HSP27) was shown in response to mechanical pressure. The pressure mediated HSP27 phosphorylation could be blocked by the p38 specific inhibitor SB203580. We could demonstrate a transient phosphorylation of MAP kinase kinases 3/6 (MKK3/6) similar to p38. Additionally, we examined the suppression of pressure-induced p38 phosphorylation after blocking protein kinase C (PKC) with calphostin C. Furthermore, the involvement of small G proteins of the Rho family in the mechanical induced signalling cascade was investigated. Inhibitor studies dealing with toxin B as well as transient transfection experiments using dominant-negative constructs of Rac showed no effects on the pressure induced p38 phosphorylation.
Our experimental results indicate mechanical pressure as a new type of cellular stress which yields in p38 activation with implications to (patho)physiological conditions as acanthosis or the Koebner phenomena.

P213

The role of I?B kinases IKK1 and IKK2 in calcium-induced differentiation and proinflammatory activation of human keratinocytes.

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Disturbed proliferation and differentiation processes of keratinocytes play a major role in the pathogenesis of many skin diseases. Intracellular signalling mechanisms which regulate the balance between epidermal proliferation and differentiation, however, are thus far largely unknown. We here intended to analyze the impact of the IKK/I?B/NF-?B signalling pathway on differentiation of keratinocytes. Primary human keratinocytes as well as HaCaT cells were retrovirally infected to express dominant negative (dn) mutants of NF-?B upstream kinases IKK1 and IKK2. Functionality of the generated mutants was subsequently confirmed by Western blot analysis of I?B degradation. Thereafter, differentiation of keratinocytes was induced by elevation of extracellular calcium levels. The differentiation state of keratinocytes was then assessed by studying morphology and expression of differentiation markers such as involucrin and keratins 1 and 10. In contrast to data reported from animal models, we could not detect any effects of mutated IKK1 or IKK2 regarding calcium-induced differentiation. However, inflammatory activation of keratinocytes as measured by TNF-a-mediated up-regulation of ICAM-1 and IL-8 was almost completely inhibited in cells expressing dn IKK2 whereas it was only partly blocked in IKK1 dn cells. In conclusion, our data suggest that, in least in vitro, IKK1 and IKK2 are not involved in the regulation of calcium-induced keratinocyte differentiation while they are pivotal for inflammatory activation.

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Influence of different ß-cyclodextrin complexes on the proliferation of HaCaT keratinocytes

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Objectives: Cyclodextrins are frequently used in transdermal therapeutic systems to increase the solubility and availability of the drugs. There is only a little known about the biostimulating and bioinhibiting effects of cyclodextrins and cyclodextrin complexes. Therefore the influence of cyclodextrin complexes with different substances on the proliferation of HaCaT keratinocytes has been studied by bioluminescence ATP measurements and fluorometric DNA quantitation.
Material and methods: Human HaCaT keratinocytes was cultured according to the common procedure during 5-7 days. After harvesting the cells their number was determined by means of cell counter CASY 1(Schaerfe System GmbH, Germany). Complexes of ß-cyclodextrin with adrenalin, theophyllin, cholesterin, paraformaldehyd, berberinchloride and tyramin hydrochloride, respectively, were used. The HaCaT cells were incubated in a 96-well microplate with these ß-cyclodextrin complexes in different concentrations (0.1 %,0.5 %,1 %) 24 h and 48 h, respectively. The determination of proliferation was carried out on the basis of the bioluminescence assay ATPLiteTM-M (Perkin Elmer Bioscience, Netherlands) using a luminometer LUMIstar Galaxy (BMG LabTechnologies GmbH, Offenburg, Germany) and by means of the fluorometric DNA quantitation using Hoechst dye 33342 performed on a fluorometer FLUOstar Galaxy (BMG LabTechnologies GmbH, Offenburg, Germany).
Results: ß-Cyclodextrin complexes in concentrations between 0.1 % and 0.5 % (incubation time 24 h) can generate a proliferation of HaCaT cells in general, only the complexes with adrenalin and theophyllin caused bioinhibiting effects already in low concentrations
The higher concentrated (1%) cyclodextrin complexes inhibited the proliferation of HaCaT cells after 48 h incubation.
Conclusions : The use of these ß-cyclodextrin complexes in concentrations lower than 0.5 % should be harmless also for applications on the skin.

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Murine ß-defensins 1, 3, 4, and 6 are expressed constitutively or after stimulation in embryonic, neonatal, and adult skin: innate immunity during development of the adaptive response.

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Antimicrobial proteins of the innate immune system are essential for cutaneous defense against invasive bacteria. Defensins comprise a family of cationic antimicrobial peptides that is characterized by the conserved 6 cysteine residues. Embryonic development occurs in an environment largely devoid in pathogens. In contrast, newborns are suddenly exposed to wide variety of environmental germs. In newborns the cellular immune system is immature, nevertheless, infections are rare. We examined whether the switch from the embryonic state, to newborn and to adult, that means the exposure to environmental noxious agents and maturation, influences murine ß-defensins 1, 3, 4, and 6 expression in the skin. Immunohistology and RT-PCR revealed constitutive expression of murine ß-defensin 1 in embryonic, as well as in neonatal (five days old) and in adult skin (four weeks old). ß-defensin 3 was not expressed constitutively, but noted after stimulation of adult mouse skin with phorbol ester PMA. ß-defensin 4 expression was found without pathogenic stimulation in embryonic and neonatal skin, but was reduced in adult skin. Murine ß-defensin 6 was described previously in esophagus, tongue, trachea and skeletal muscle (Yamaguchi et al., 2001). Using RT-PCR we showed for the first time that ß-defensin 6 is expressed in the skin, but only in adult mice. We suggest that the natural microflora of the skin may induce transcription of that defensin. In summary, we showed that the expression of murine defensins depends on exposure to environmental pathogens, on maturation, and on stimulation. Antimicrobial peptides may provide a compensatory innate defense to the development of cellular immune response mechanisms in the newborn period.

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NF?B promotes UVB-induced apoptosis by downregulation of TRAF-proteins

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Activation of the transcription factor nuclear factor-?B (NF?B) via interleukin 1 (IL-1) is generally associated with the induction of antiapoptotic pathways within the cell. Accordingly, NF?B was shown to suppress death ligand-induced apoptosis via transcriptional upregulation of antiapoptotic proteins IAPs (inhibitor of apoptosis proteins) and FLIP (FLICE inhibitory protein). In contrast, we could demonstrate that UVB-mediated cell death was significantly enhanced upon NF?B activation. Enhancement of UVB-induced apoptosis coincided with a release of about 100 pg/ml of the proapoptotic cytokine tumor necrosis factor-a (TNFa). Surprisingly, costimulation of cells with the same dose of TNFa and UVB or application of supernatants of IL-1 plus UVB-treated cells to UVB-irradiated cells resulted only in a rather weak enhancement of UVB-induced apoptosis, indicating that other intracellular components have to be involved in the proapoptotic effect of NF?B. Gene chip arrays revealed that, in contrast to the NF?B-dependent transcriptional up-regulation of TNFa, NF?B-dependent transcription of c-IAP and FLIP was completely inhibited by UVB radiation. In addition, members of the TRAF (tumor necrosis factor receptor-associated factor) protein family which contain NF?B consensus elements within their promoters were shown to be significantly downregulated upon UVB exposure. TRAF proteins are upstream adapter proteins associated with the cell death receptor TNFR-1. Upon activation by TNFa, the recruited TRAF proteins transduce antiapoptotic signalling pathways mediating cell survival. In parallel, the proapoptotic adapter protein FADD can be recruited to TNF receptor-1, resulting in induction of apoptotic cell death. Taken together, activation of NF?B in association with UVB exposure results in upregulation of the proapoptotic death ligand TNFa. In addition, it results in transcriptional inhibition of the antiapoptotic cIAP and FLIP genes and of the TNF receptor-1 related antiapoptotic adapter proteins TRAFs, thereby promoting UVB-induced apoptosis. These findings explain why activation of NF?B, in contrast to other apoptosis models, does not protect from but even enhances UVB-induced apoptosis.

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Interleukin-6-type cytokines upregulate expression of multidrug resistance-associated proteins in NHEK and dermal fibroblasts

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Normal human epidermal keratinocytes (NHEK) and dermal fibroblasts have been shown to express a cell-type specific pattern of influx and efflux transport proteins as well as extrahepatic cytochrome P450 enzymes showing that these cells metabolize and excrete a variety of xenobiotics like drugs. In this project we analyzed the influence of IL-6-type cytokines on the expression of multidrug resistance-associated proteins (MRP1-6). Using RT-PCR, real-time PCR, cDNA microarrays, immunostaining and efflux assays we were able to show that stimulation of NHEKs and human dermal fibroblasts with IL-6, particularly in combination with its soluble receptor, or oncostatin M for 48 hours resulted in an upregulation of MRP-expression and activity. It is well known that several inflammatory skin diseases like psoriasis show an enhanced expression of IL-6-type cytokines. Correspondingly, upregulation of MRP-expression was found in skin lesion taken from patients with psoriasis and lichen ruber. Interestingly, MRP6 expression could be upregulated in keratinocytes of patients with pseudoxanthoma elasticum by IL-6 treatment which may lead to new concepts for the treatment of this heritable disease. Using the MEK1-specific inhibitor UO126 we can demonstrate that these effects seem to be independent of the activation of MAPK belonging to the Erk1/2-family, suggesting a potential role for the Jak/STAT-pathway.

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Ratiometric determination of changes in membrane potential in melanoma cells by means of flow cytometry

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To determine the membrane potential of human cells by flow cytometry, the anionic oxonol DiBAC4(3) has often been applied. Although the quantitative uptake of the oxonol into the cells is dependent on membrane potential, there is a binding to intracellular proteins too. Cells being in different phases of the cell cycle consistently possess different amounts of protein, so that ratiometric approaches are absolutely necessary to compensate for protein-bound fluorescent dye.
The present investigation should test the approach of Seamer and Mandler [1], who suggested the use of the side scatter height (SSC-H) as a parameter for compensation. First, a high correlation between SSC-H and protein content of single cells could be demonstrated using CellTracker GreenBODIPY (Molecular Probes) for protein staining. The changes in membrane potential between untreated and cell cycle synchronized IGR-1 melanoma cells (the latter had a high proportion of cells being in the S and G2/M phase) was apparently very high without compensation. After calculating the ratio of DiBAC4(3) and SSC-H, the increase in membrane potential dropped from about 70 % to 13 % and was then comparable to values measured electro-physiologically [2]. As a result, the use of SSC-H for ratiometric determination of changes in membrane potential seems to be valid.

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P219

Increased Epidermal Ceramide Content in Aged Mouse Skin as Shown by Electrospray Ionisation Mass Spectrometry

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Lipids are important for the cellular structures of the stratum corneum and cell membrane, partnering with corneocytes to enable barrier function of the skin. Lipids are also significant in signal transduction for growth, differentiation and apoptosis. The composition and the amount of individual lipids are of considerable importance for skin homeostasis. Alterations in specific lipid patterns may play an important role in health and disease of the skin. The aims of the present study were to establish the suitability of electrospray ionisation-tandem mass spectrometry (ESI-MS) for direct analysis of different lipid species, in particular ceramides and phosphatidylcholine in crude lipid extracts from mouse skin, and to compare lipid composition in aged versus young skin. The lipid species were identified on the basis of their characteristic mass to charge ratio (m/z value), fragmentation analysis, parent ion scan and fatty acid scan. We found changes in epidermal lipid composition in young (< 3 month) versus aged (> 18 month) mice. Both groups showed a considerable diversity in lipid composition, but in aged mice, we found an even broader distribution of several lipid species compared to young mice. Not only the composition but also the relative amount of lipids in the epidermis from aged mice were significantly altered in comparison to those from young mice. Aged mouse skin contained relatively more ceramides whereas in young mouse skin phosphatidylcholine and sphingomyelin species dominate the lipid composition. The relative amount of phosphatidylcholines and sphingosines (measured as m/z intensity) with acyl chain length between C16 and C24 was found to be four to five times higher in young mouse skin compared to aged. The latter, surprisingly, contained eight times more ceramides, mainly long chain fatty acid species, than found in young epidermis. In summary, we found that ESI-MS techniques such as lipid-species specific parent ion scans combined with fatty acid scans provides a convenient tool extending our knowledge of lipid composition and lipid alterations in pathophysiological and physiological states of the skin.

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Endothelial vascular endothelial growth factor receptor-2 expression is subject to regulation by distinct small Rho GTP binding proteins

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Recent evidence suggests that Rho-family small GTPases play a significant role in the regulation of different endothelial cell functions, implicated in capillary network formation. Whereas Rho-family small GTPases are primarily known to control actin-based motility processes, RhoA, Rac1, and Cdc42 have been shown to regulate other cellular activities as well, such as membrane transport and gene transcription. As vascular endothelial growth factor receptor-2 (VEGFR2) expression is necessary for angiogenic responses to occur, we hypothesized that Rho-family small GTPases may affect VEGFR2 expression by cultured endothelial cells (ECs). Overexpression of dominant negative mutants N17Rac1 and N19RhoA were seen to significantly inhibit VEGFR2 protein expression by HUVEC, whereas transfection of mutant N17Cdc42 failed to affect VEGFR2 levels. As N17Rac1 and N19RhoA also suppressed VEGFR2 mRNA accumulation, we subsequently examined their effects on VEGFR2 transcriptional activation. Analyses of a different 5'-deletional VEGFR2 promoter-based reporter gene constructs revealed that inhibition of N17Rac1 and N19RhoA is conveyed by distinct gene-regulatory elements. Whereas N17Rac1-mediated suppression is confined to a GC-rich region between bp -77 and -60, N19RhoA-mediated inhibition appears to be conferred by an element located between bp -225 and -160, harboring a consensus E-box binding site. Therefore, different members of the Rho-family small GTPase family exert diverse effects on VEGFR2 expression. Significantly, inhibition of VEGFR2 transcription by N17Rac1 and N19RhoA involves distinct molecular mechanisms as different elements seem to be engaged. In conclusion, control of capillary network formation by Rho-family small GTPases may likely be mediated in part via modulation of VEGFR2 expression

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Control of the ß2-adrenergic signal by tetrahydrobiopterin

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Previously it has been reported that human proliferating/undifferentiated basal keratinocytes hold the full capacity for autocrine catecholamine synthesis and express ß2-adrenoceptors (ß2-AR). Only very recently, we found evidence that also human melanocytes produce catecholamines and express ß2-ARs. It is well established that the a-MSH/ MC-1 receptor system stimulates melanogenesis through the effect of cyclic AMP (cAMP). Since stimulation of ß2-ARs also yields an increase in cAMP, these findings add a new source to the pigmentary response in human skin. Both cAMP and 6-tetrahydrobiopterin (6BH4) play important roles in the expression and regulation of the ß2-AR signal. Moreover, cAMP together with its response element (CRE) controls the transcription of (a) GTP cyclohydrolase I, the rate limiting enzyme for the de novo synthesis of 6BH4, (b) tyrosine hydroxylase (TH) the rate limiting enzyme for the catecholamine synthesis and (c) ß2-AR transcription. Hence, 6BH4 controls the synthesis of epinephrine via TH, and in turn epinephrine determines ß2-AR densities. Here we asked the question whether 6BH4 could possibly directly control the ß2-AR by binding to this 7 TM|- protein. Using [3H] 6BH4 and ß2-AR we were able to identify one 6BH4 binding site on the receptor. Functionality of this binding was followed with the cAMP response and receptor density in human keratinocytes and endothelial cells (HUVEC). The results clearly showed that 6BH4 enhanced both cAMP production as well as receptor numbers. HyperchemTM molecular modeling with a deep view revealed that binding of 6BH4 to the intracellular loop 3 yields a dramatic change of the structure to an exact ß-pleated sheet. Taken together, these results lead to a new fundamental understanding in the catecholaminergic signaling cascade and open new therapeutic dimensions.

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Vanilloid receptor 1 expression in the human skin suggests multiple functions beyond sensory nerve signalling

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Vanilloid receptor 1 (VR1), a key molecular integrator of nociceptive sensory mechanisms, is also expressed by and functionally active in cultured human epidermal keratinocytes. In this study, using immunohistology, RT-PCR and functional assays, we wished to further characterize VR1 expression and its functional characteristics in other cell types of human skin. Within the skin epithelium, VR1 immunoreactivity (ir) was localized to the epidermis (predominantly to its basal layers), to the inner and outer root sheaths (ORS) and matrix keratinocytes of the hair follicle (HF), sweat gland epithelium, and sebocytes, but was absent from the follicular dermal papilla. Furthermore, strong VR1-ir was also observed on smooth muscle and endothelial cells of blood vessels, and on mast cells and dendritic cells (as revealed by double staining with cell-specific markers). Epidermal melanocytes and dermal fibroblasts showed no VR1-ir. By RT-PCR, microdessected human scalp hair follicles transcribed the VR1 gene. Since HF provide an excellent model for exploring the effects of a test agent on epithelial proliferation and differentiation in an epithelial-mesenchymal interaction context, we also investigated the effects of VR1 ligands (vanilloids) on human hair growth parameters in vitro. VR1 stimulation by capsaicin inhibited hair shaft elongation, which could largely be abrogated by the VR1 antagonist I-RTX. Furthermore, capsaicin treatment induced apoptosis and catagen transformation, and decreased the number of proliferating cells in the hair bulb. In addition, capsaicin inhibited proliferation and stimulated both differentiation and apoptosis of VR1-expressing, cultured ORS and HaCaT keratinocytes in a VR1-mediated fashion. In summary, these data strongly suggest that the cutaneous expression of VR1 serves multiple functions beyond sensory nerve signalling, and raise the question which endogenous VR1 ligands are employed by human skin for differential signalling through this receptor on various cell types.

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Effects of Cytochrome P450-Derived Epoxides on Transglutaminase Expression and Activities in Epidermal Keratinocytes

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Cytochrome P450 CYP2B19 is a keratinocyte-specific epoxygenase, which generates epoxyeicosatrienoic (EET) acids from arachidonate. In recent studies, the CYP2B19 product 14,15-EET activated transglutaminase activities and induced cornification of mouse and human epidermal keratinocytes. Present studies aim to elucidate the mechanisms and transglutaminase gene products responsible for the 14,15-EET-induced increase in cross-linking activities. This study addressed whether increased steady state transglutaminase mRNA levels are associated with 14,15-EET-induced transglutaminase activation. Human epidermal keratinocytes were induced to differentiate, and treatments were added to the medium daily for 4 days (complete Keratinocyte Growth Medium, 1.4 mM Ca++). Messenger RNA was isolated, and semi-quantitative RT-PCR was used to evaluate effects of 14,15-EET (2 µM), vehicle (0.1% ethanol) or cholesterol sulfate (15 µM) on transglutaminase mRNA levels. Differentiating cultures expressed detectable levels of transglutaminase II and the differentiation-associated transglutaminases (I, III, V) implicated in cornified cell envelope formation. Next, we assessed treatment effects on mRNAs encoding the differentiation-associated transglutaminases. cDNA templates were amplified over a range of cycle numbers that generated increasing product. After 1, 2, 3, or 4 days of treatment, variations in cDNA product formation were similar among all three treatments. The duration of in vitro differentiation was the main variable affecting cDNA product formation, which increased over days 1-3. Similar results were obtained for types I, III, and V mRNAs. Western blot data corroborated results for transglutaminase I. The B.C1 monoclonal antibody detected a ~105 kDa protein, which had similar signal intensities in 14,15-EET and vehicle treated cultures. Signal intensities increased during in vitro differentiation (Days 0-6), regardless of treatment. These results suggest that the effects of 14,15-EET on keratinocyte transglutaminase activities may involve post-translational, rather than transcriptional, regulatory mechanisms.

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Inhibition of NF-kB downregulates cIAP2 and sensitizes human keratinocytes to tumor necrosis factor (TNF) a-induced apoptosis

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Tight regulation of apoptosis is crucial for the maintenance of tissue homeostasis in the skin. Death ligands like TNFa and *TNF-related apoptosis inducing ligand* (TRAIL) activate not only an intrinsic death program but also regulate other signalling pathways implicated in proinflammatory responses. While TRAIL and TNFa are both able to induce NF-?B in human keratinocytes, only TRAIL but not TNFa potently induces apoptosis. Interestingly, when NF-kB activation was inhibited by retroviral infection of keratinocytes with a *kinase dead IKK2 mutant* (HaCaT-IKK2-KD), TNFa-induced but not TRAIL-induced apoptosis was dramatically enhanced. Susceptibility to TNFa-induced apoptosis was neither explained by differences in cell surface expression of TNF receptors nor by expression of the initiator caspases 8 or 10. Biochemical characterization revealed that sensitization to TNFa-induced apoptosis in HaCaT-IKK2-KD occurred at the level of caspase 8 activation. Accordingly sequential retroviral infection of the caspase 8 inhibitor $cFLIP_L$ fully inhibited TNFa-induced cell death in HaCaT-IKK2-KD. To further investigate the susceptibility to TNFa in HaCaT-IKK2-KD, we analyzed a panel of NF-?B-regulated antiapoptotic effector molecules. Interestingly, the *inhibitor of apoptosis* (IAP) family member cIAP2, but not cIAP1, XIAP or TRAF2 was specifically downregulated in HaCaT-IKK2-KD on the mRNA and protein level.
In conclusion, we demonstrate that inhibition of NF-kB dramatically sensitizes human keratinocytes to TNFa-mediated apoptosis. This sensitization occurs at the level of caspase 8 activation and may critically involve the NF-kB target gene cIAP2. Our data suggest that cIAP2 might deviate the biological outcome of TNF receptor stimulation to a proinflammatory response in human skin.

P225

A serine protease mediates C-terminal cleavage of the 120 kDa soluble ectodomain of type XVII collagen/BP180 (LAD-1) resulting in a 97 kDa polypeptide (LABD 97)

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Type XVII collagen/BP180 is a hemidesmosomal transmembrane glycoprotein of basal keratinocytes. Several subepidermal blistering diseases are associated with an autoimmune response to this molecule, including linear IgA disease (LAD). Major antigenic targets in LAD are 2 proteolytic fragments of type XVII collagen: an epidermis-derived 97 kDa protein (LABD 97) and a keratinocyte-derived 120 kDa polypeptide (LAD-1). The N-termini of both fragments localize within the membrane-proximal 16th non-collagenous A (NC16A) domain [amino acid (aa) 490 to 565] of type XVII collagen. An additional cleavage at the C-terminus of Type XVII collagen, that occurs around aa position 1300, results in LABD 97. While it has been demonstrated that the N-terminal cleavage of type XVII collagen is mediated by ADAMs (a disintegrin and metalloprotease), the present study addressed the question which protease(s) cleave(s) at the C-terminus. In a first set of experiments, we observed that LAD-1, obtained from the supernatant of cultured keratinocytes may degrade to a 97 kDa polypeptide. This keratinocyte-derived 97 kDa polypeptide, like epidermis-derived LABD 97 and the 120 kDa LAD-1, reacted with IgA autoantibodies from LAD patients´ sera. The 97 kDa keratinocyte-derived polypeptide was also recognized by polyclonal antibodies against the NC16A domain, indicating that the polypeptide was produced by C-terminal degradation of LAD-1. From these results, we concluded that the 97 kDa fragment is a keratinocyte-derived equivalent of epidermal LABD97. Interestingly, the C-terminal degradation was only observed when the LAD-1-rich fraction was prepared using culture medium from keratinocytes that had been plated more than 3 days ago. Moreover, this degradation was abolished by serine protease inhibitors. These results demonstrate that the protease mediating the C-terminal cleavage of LAD-1 is a serine protease(s) secreted by keratinocytes. Further studies will attempt to purify this protease from the culture medium.

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Angiotensin Converting Enzyme is Regulated in Human Dermal Microvascular Endothelial Cells by Ultraviolet Irradiation, Cytokines and Sensory Neuropeptides.

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Ultraviolet- (UV)-irradiation of the skin results in various pro- as well as antiinflammatory effects and the induction of apoptosis in epidermal or dermal cells. Human dermal microvascular endothelial cells (HDMEC) respond to UVB with increased expression of cell adhesion molecules, cytokines, and proopiomelanocortin. The zinc metalloprotease angiotensin converting enzyme (ACE) plays a substantial role in the control of systemic blood pressure and renal function. In the skin, recent evidence suggests that lack of functional ACE promotes inflammation presumably by increasing the substance P (SP) and bradykinin (BK) bioavailability. In this study we address the hypothesis that endothelial ACE expression is regulated in response to UVB and inflammatory mediators in vitro. HDMEC were treated with UVB-light (5 - 30 mJ/cm^2, SP, IL-1, IL-6, TNFa or IFN? for 1 - 48 h. Subsequently, quantitative RT-PCR was performed using primers specific for ACE. After 1 and 3 h, UVB irradiation, SP (10 & 100 nM), cytokines (TNFa, IL-1, IFN?) significantly decreased constitutive mRNA levels of ACE. HDMEC flow cytometry analysis confirmed that ACE cell surface expression was downregulated as early as 3 h, but in particular 24 h and 48 h after UVB in a dose-dependent manner. In addition, TNFa, IFN? and particularly IL-1, but not IL-6 substantially reduced HDMEC ACE expression. These data indicate that sensory neuropeptides, proinflammatory mediators and UVB light, directly or indirectly presumably via cytokines such as IL-1 are capable of regulating ACE expression in HDMEC. Downregulation of ACE during skin inflammation may result in a temporally increased susceptibility of these cells to peptide mediators such as SP or BK and a reduced generation of the vasoconstrictor angiotensin 2. This may have a significant role in controlling local cutaneous inflammatory responses as well as vascular tone and blood pressure.

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Differential expression of renin, angiotensinogen and angiotensin-converting enzyme in psoriasis and basalioma

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We were recently able to describe the expression of a complete renin-angiotensin-system (RAS) in human skin and to show the ability of keratinocytes to synthesise angiotensin II. A complete RAS comprises the precursor molecule angiotensinogen as well as the enzymes (renin and angiotensin-converting-enzyme [ACE]) required for cleavage of angiotensinogen to generate the main effector hormone angiotensin II. Angiotensin II not only exerts its well known cardiovascular actions, but also regulates cell proliferation and differentiation in various tissues. First data concerning human skin show a proliferative effect on keratinocytes.
The present study represents a first approach to examine a putative involvement of the RAS in benign or malignant dermatoses coinciding with hyperproliferation by looking at putative alterations in the expression of RAS components in psoriasis (representing benign hyperproliferation) and basalioma (representing malignant hyperproliferation). Sections of normal or affected human skin were forwarded to immunohistochemical stainings using specific antibodies directed against human angiotensinogen, renin or ACE, respectively. While in normal skin all antibodies produce a homogenous, positive signal throughout the whole epidermis, the staining pattern is altered in both dermatoses examined. In basalioma, tumor cells stain less intensely than the epidermis with the exception of tumor cells in areas of strong proliferation, which are the edges of the tumor as well as smaller tumor satellites in deeper dermal layers. Epidermal areas in close association with the tumor also stain less intensely. In psoriasis, strong signals for all components examined can be found in basal epidermal layers and the stratum granulosum, while – in contrast to normal skin - the stratum spinosum shows almost no labelling with all antibodies used.
The alterations in the expression of RAS components in basalioma and psoriasis suggest that the RAS is involved in the pathophysiology of benign and malignant hyperproliferative dermatoses. The exact mechanisms involved remain to be elucidated in future studies.

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Interaction of tight junctions with inflammatory cells in the epidermis

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Tight junctions are well known as barrier forming cell cell junctions in simple epithelia and endothelia. Recently these structures have also been identified in murine and human skin and various reports suggest that they play an important role in barrier function of the epidermis. As TJ have also been shown to interact with inflammatory cells in simple epithelia and endothelia, we asked whether the same might be true in the epidermis. We investigated a panel of TJ transmembrane and plaque proteins that are known to be important in cell cell interaction as well as in function, assembly, and/or regulation of TJ by using immunofluorescence microscopy. Interestingly we observe a down regulation of TJ proteins in various diseases, e.g. psoriasis, lichen ruber and ekzema, in areas where inflammatory cells pass though, while they are upregulated in other areas, e.g. without strong inflammation. Moreover we show in some cases a resealing of TJ after inflammatory cells passed through. As it is known that the various cell cell junctions are interconnected and an opening of all cell cell junctions should be necessary for the migration of inflammatory cells in the epidermis, we also investigated marker proteins of desmosomes, adherens junctions and gap junctions, and discuss a temporal relationship of the opening of cell cell junctions. Our results argue for an interaction of TJ and inflammatory cells in the skin. The molecular mechanism of this interaction has to be clarified in future experiments.

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Down regulation of c-kit proto-oncogene and Microopthalmia transcription factor in human mast cells and melanocytes using siRNA technology

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The SCF receptor is encoded by the proto-oncogene c-kit. Ligation of the c-kit receptor by SCF induces its dimerization, which leads to proliferation and activation of mast cells and maturation of melanocytes. The c-kit gene promoter contains a functional binding site for the micro-ophthalmia transcription factor (Mitf), which specifically regulates c-kit expression in mast cells and melanocytes. Knockout of c-kit genes in transgenic mice causes the complete absence of mast cells.
In the present study, we transfected human dermal mast cells, primary human melanocytes and HMC-1 cells (mast cell leukaemia cell line) with c-kit and Mitf targeted siRNA (small interfering RNAs) and DNA oligonucleotides (for endogenous siRNA synthesis). For transient silencing of the Mitf and c-kit gene, respectively, we designed 21 nucleotide double stranded siRNAs corresponding to gene position 663, which is a transactivation domain for all isoforms of Mitf and, additionally, siRNAs corresponding to gene position 762, which represents an extracellular domain of the c-kit gene. In order to achieve a stable down regulation of the c-kit and Mitf genes, we cloned DNA oligonucleotides corresponding to gene position 629 of the c-kit and to gene position 141, exon 1, of Mitf into the P-suppressor-Neo-vector, which contains an U6 promoter. The cloned vectors were transfected into the cells by lipid transfection reagent. To obtain siRNA positive cells, the cells were treated with geneticin.
Western-Blot analysis of dermal mast cells, HMC-1 and melanocytes showed a distinct down regulation of Mitf 48 hours after transfection with siRNA. FACS analysis of c-kit expression in mast cells revealed a 50 percent down regulation of the receptor, indicating that Mitf is an essential transcription factor for the expression for the c-kit gene. Silencing of c-kit in dermal mast cells down regulated protein expression down to 30 percent depending on the siRNA concentration. FACS analysis of the transiently transfected cells showed a significant decrease of cell viability.
Our data suggest that RNA silencing may be a suitable approach for a stable or transient downregulation of c-kit and Mitf in mast cells and melanocytes.

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Laser capture microdissection in the analysis of a mouse model for epidermolytic hyperkeratosis

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The use of laser capture microdissection (LCM) has been a major step towards a better understanding of the molecular mechanisms that play a role in various disease processes. To study genetic changes that occur in a particular cell type, tiny subpopulations of cells need to be isolated to reduce the risk of contamination. The skin is an attractive organ for the application of LCM since it is composed of a complex mixture of different cell types. Here we describe the use of LCM in the analysis of an animal model for a hereditary blistering disorder, epidermolytic hyperkeratosis (EHK) that allows induction of the phenotype in a cicumscribed area of the skin. "Activation" of a keratin 10 point mutation in a circumscribed area of the skin generated two genetically different populations of keratinocytes. Microdissected keratinocytes from persisting hyperkeratotic lesions were a source of genomic DNA and were further subjected to PCR analysis. Cells from surrounding, phenotypically normal skin served as a control. We demonstrate that LCM has proven a valuable and reliable tool in the isolation of pure samples of keratinocytes from different areas of mouse skin. The information obtained by LCM provided new insights into the molecular and cellular basis of mosaic skin disorders.

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Inner ear melanocytes are sensitive to irradiation with UVB

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Pigmented cells in the inner ear containing melanin have been identified a long time ago and been called melanocytes or melanocyte like cells. However not much is known about these cells due to their difficult accessibility and the lack of cell culture methods. We describe here the first successful cultivation of inner ear melanocytes and their characterization as "true" melanocytes with various markers known from epidermal melanocytes, e.g. tyrosinase, Melan A and Mel 5. In cell culture, inner ear melanocytes grow more slowly than epidermal melanocytes and show a strong dependence on fibroblasts concerning growth and morphology. Surprisingly, even though UVB-light is normally absent in the inner ear, UVB-irradiation of inner ear melanocytes resulted in an elevated production of melanin in some cultures. Summarizing our results we demonstrate that inner ear melanocytes show similarities and differences to epidermal melanocytes. The now provided possibility to culture inner ear melanocytes will help to investigate these cells more in detail, probably elucidate additional functions of melanocytes and will enlarge the knowledge of melanocytes in general.

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Human Thy-1 (CD 90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18)

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The adhesion of circulating cells to endothelial cells plays a central role in inflammation as well as in the metastasis of tumor cells. Leukocyte recruitment in response to inflammatory signals is in part governed by interactions between endothelial cell receptors belonging to the immunoglobulin superfamily on one side and leukocyte integrins on the other.
Previously, the human glycoprotein Thy-1 (CD90) was identified as an activation-associated cell adhesion molecule on human dermal microvascular endothelial cells (HDMEC). There were evidences, that the leukocyte integrin Mac-1 (CD11b/CD18) is a receptor for the human Thy-1 on activated HDMEC. Antibodies against CD11b were capable of reducing the binding of purified Thy-1 to CD11b-expressing monocytes, polymorphonuclear cells (PMNC) as well as to CD11b-transfected CHO cells.
Now, a direct interaction of the Mac-1- and Thy-1-protein in a cell-free system was demonstrated. Further, leukocyte adhesion to activated endothelium as well as the subsequent transendothelial migration was shown to be mediated by the interaction between Thy-1 and Mac-1.
The interaction between Mac-1 and ICAM-1 is supposed to be a predominant adhesion pathway mediating the firm adhesion of leukocytes to endothelial cells at sites of inflammation, since the expression of ICAM-1 is induced or increased upon cell activation by pro-inflammatory mediators. But, the presence of additional leukocyte-endothelium adhesion pathways mediated by interaction of Mac-1 (CD11b/CD18) with a ligand distinct from ICAM-1 was suggested in different studies using ICAM-1 deficient mice or antibody blocking experiments.
The data presented, prove that the human Thy-1 is a second endothelial cell receptor for the leukocyte integrin Mac-1, that contributes to leukocyte recruitment to sites of inflammation, tissue injury or infection. Consequently, further investigations aimed at the molecular biology as well as the regulation of leukocyte emigration into perivascular tissue have to include the Thy-1/Mac-1 interaction as well, besides well characterised adhesion processes.

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Lysyl hydroxylase 2 - key enzyme for hydroxylysine-derived collagen cross-links

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Sclerotic skin diseases (keloids, systemic scleroderma, lipodermatosclerosis) are characterised by an increase of hydroxylysine derived collagen cross-links, which are virtually absent in normal skin. These cross-links require a hydroxylysine residue located in the telopeptides of the collagen molecule. Recently, it has been shown that lysyl hydroxylase 2 is a telopeptidyl lysyl hydroxylase in osteoblasts. In the present study, we analysed the effect of an overexpression of LH-2 on the cross-link pattern of collagens synthesised by human dermal fibroblast in long term culture.

Lysyl hydroxylase 2b containing exon13A was cloned into a recombinant adenoviral cosmid vector (pAdcos45) basing on adenovirus type 5. LH2b was transient transfected into confluent normal human foreskin fibroblast. After 4 weeks cells and cell associated extracellular matrix were scraped off and processed for further analysis. Collagen cross-links were analysed after borohydride reduction and collagenase digestion of the synthesised matrix. The level of steady state mRNA of collagen I and LH-2 was analysed by real time PCR.

Overexpression of LH-2 resulted in an increase of hyl-derived and a decrease of lys-derived collagen cross-links (lys-derived: control 0.19 ± 0.02, transfected 0.08 ± 0.01 mol/mol, $p<0.001$; hyl-derived: control 0.12 ± 0.03, transfected 0.17 ± 0.01 mol/mol, $p=0.007$). This increase was accompanied by an increase of collagen synthesis (control: 940 ± 186 pmol, transfected 8100 ± 3500 pmol, $p=0.004$). Real time PCR showed a marked increase of the transcriptional activity of LH 2 (+500%), while the steady state mRNA level of collagen I was only slightly increased (+100%).

Our results indicate that lysyl hydroxylase 2 is responsible for the hydroxylation of lysine residues located in the telopeptides of the collagen molecule. Furthermore, an overexpression of lysyl hydroxylase 2 resulted in a marked increase of collagen synthesis revealing a new function of this enzyme. Therefore, lysyl hydroxylase 2 might be a target for a putative therapy of sclerotic skin diseases.

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The Murine Dermis Comprises Cells with Hematopoietic Clonogenic Potential

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Evidence exists that the dermis contains cells which have the capacity to differentiate into multiple cell types, suggesting that there may be a multipotent stem cell type, the phenotype of which is still unclear. We wanted to elucidate whether the murine dermis contains hematopoietic stem cells (HSC) and if so to identify them. Flow cytometry analysis revealed that 10% and 4% (n=5) of adult and newborn dermal mouse cells, respectively express CD34. The overwhelming majority (99%) of these CD34+ cells failed to express CD45 antigens. However, coexpression of CD45 by less than 1% of CD34+ cells within adult and newborn dermal cells and coexpression of CD45 by less than 1% of Sca-1+ and CD117+ cells suggests the presence of a small reservoir of progenitor cells or even HSC within mouse dermis. When testing the in vitro clonogenic capacity of either unpurified dermal cells or lineage (Lin)-depleted dermal cell populations enriched for CD45, Sca-1, and CD117 in hematopoietic colony-forming assays, we found that all populations produced cells of myeloid lineages. Experiments evaluating the in vivo hematopoietic reconstitution potential of enriched (e.g. Lin-Sca-1+, Lin-CD34+) dermal cell fractions are in progress. Given its easy accessibility, our further description of the dermis as a potential source of extramedullary hematopoietic stem cell activity makes it an attractive alternative for blood stem cell therapeutics.

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Phosphatidylcholine-sphingomyelin transacylase in epidermal permeability barrier formation

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As de novo synthesis cannot provide membrane phospholipids with sufficiently defined fatty-acids, mammalian cells remodel their fatty-acid composition by deacylation/reacylation. We found that human keratinocyte plasma membranes contain an enzymatic activity that transfers acyl-chains from phosphatidylcholine (PC) to sphingomyelin (SM). The term transacylase fits the yet undescribed activity, as the reaction does not produce free fatty-acids nor deacylated PC and SM molecules.An epidermis-specific function of the PC-SM transacylase could reside in permeability barrier formation. A signifcant part of stratum corneum lipids is formed by ceramides. These ceramides differ in their type of sphingoid base as well as in chain-length and saturation of esterified fatty-acids. Some Cer-species contain the double-unsaturated linoleic acid ester-linked to omega-hydroxy fatty-acids. Substitution of oleate for linoleate in these special Cer-molecules results in a profound barrier abnormality. Glucosylceramides are the major precursors of stratum corneum ceramides. However, a fraction of ceramides is derived from SM. These SM-derived Cer-species are required for normal permeability barrier function.Compared to the variety of fatty-acids present in glycerophospholipids, the fatty-acid composition in SM is uniform. Mammalian SM molecules mainly contain saturated acyl-chains. The availability of linoleic acid as a free fatty-acid seems to be of secondary importance for the rate of its incorporation into stratum corneum ceramides. We suggest that the new PC-SM transacylase is involved in tuning up the fatty-acid profile of SM for later use in stratum corneum ceramides. Modulation of the transacylase activity could emerge as a potential target for treatment of permeability disorder as in atopic dermatitis.

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Functions of Ras/Rac signaling in epidermal keratinocytes

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Epidermal morphogenesis and wound repair critically depend on the modulation of keratinocyte shape. We have previously shown that the product of the H- ras oncogene is an important regulator of the formation of membrane protrusions and thereby influences keratinocyte shape. In addition, activated H- Ras protein accelerates epidermal wound healing in vitro. Using retroviral infection of primary human keratinocytes and a novel, highly efficient electroporation technique for primary cells we have analysed the ability of various Ras dependent signaling pathways to mediate keratinocyte shape changes. Using different mutants of H- Ras, Rac1 and Arf-6 as well as specific inhibitors we provide direct evidence that effects of H- Ras on keratinocyte spreading are mediated by the small GTPase Rac1, a member of the Rho family of GTP binding proteins. The ability of H- Ras to influence keratinocyte shape and to stimulate wound healing partly correlates with signaling to Rac1. Using mutants of Rac1 that interfere with its signal transduction to downstream signaling molecules we obtained first evidence for an involvement of different effector pathways in Rac1 dependent regulation of keratinocyte shape. In order to gain deeper insight into Rac1 functions we have generated transgenic mice that carry activating and inhibitory mutants of Rac1 in the epidermis. We are now using these mice to analyse Rac1 functions in murine epidermis in vivo.

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Deletion of the distal C-terminus of BP180/collagen XVII perturbs the physiologic adhesion functions

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As hemidesmosomal transmembrane component BP180 / collagen XVII is involved in the connection of the epidermis to the underlying basal membrane. Defects in collagen XVII molecule are associated with epidermal detachment in junctional epidermolysis bullosa. Here we analysed the biologic consequences of a spontanious truncation of the distal C-terminus of collagen XVII in a patient with junctional epidermolysis bullosa. The underlying mutation was a homozygous duplication of four nucleotides in exon 54 of the COL17A1 gene. It resulted in a frame shift, an adjacent nonsense sequence of 18 amino acids, and a premature termination and elimination of 43 most distal amino acids of collagen XVII. We assessed the shedding behaviour, the folding and stability of wild-type and mutated collagen XVII. IF staining with antibodies to collagen XVII endodomain and the NC16A domain resulted in a weaker signal in EB skin. Staining with the Ecto-5 antibody, which is directed against the distal 50 amino acids of the collagen XVII molecule revealed no cell membrane associated signal. Immunoblot analysis demonstrated that both truncated collagen XVII forms, the membrane-bound form as well as the shed ectodomain, were found in EB keratinocyte lysates and culture media, but in lower concentrations compared to normal cells. Analysis of the thermal stability by limited trypsin digestion showed that the helix-to-coil transition temperature midpoint of mutated collagen XVII was significantly lower, indicating abnormal folding of this molecule. These findings show that the truncation of the distal extracellular domain of collagen XVII causes abnormal folding and susceptibility to degradation, and thus perturbs the physiologic adhesion of collagen XVII to extracellular basement membrane structures and destabilizes the junctional connection.

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Evidence for expression of multiple members of the family of suppressors of cytokine signaling in normal and transformed human melanocytes

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Suppressors of cytokine signaling (SOCS) define a newly identified family of negative regulators of cytokine signaling. At present, this family consists of 8 structurally related members, SOCS-1 to -7, and CIS. We recently reported on the expression of SOCS-1 in melanoma cells in vitro and in situ. Here we investigated the expression of additional members of the SOCS family in normal human melanocytes (NHM) and 8 melanoma cell lines by multi-probe ribonuclease protection assay. CIS, SOCS-5 and -7 were similarly expressed in all of the melanoma cell lines and NHM. RNA levels of SOCS-6 were significantly higher in NHM compared with all melanoma cell lines. Most interestingly, SOCS-3 expression was absent in NHM. In most melanoma cell lines (6 out of 8) SOCS-3 was highly expressed while in 2 melanoma cell lines SOCS-3 expression was marginal. To assess the relevence of these findings, interleukin 6 (IL-6) sensitivity of the studied cell lines was determined by proliferation assays. In contrast to melanoma cell lines expressing high levels of SOCS-3, the two melanoma cell lines with marginal SOCS-3 expression were highly sensitive to IL-6. On the protein level SOCS-3 expression was absent in NHM as shown by Western immunoblotting. In melanoma cell lines SOCS-3 protein expression correlated with expression of Pim-2, a serine/threonine kinase previously implicated in stability and phosphorylation of the SOCS-1 protein. In summary, our findings, for the first time, show that multiple members of the SOCS family are expressed in human pigment cells. SOCS-3 gene expression tightly correlates with IL-6 sensitivity in melanoma cells in vitro. Further studies are needed to determine if SOCS-3 RNA knock-out can restore IL-6 sensitivity, and if SOCS-3 is expressed in melanoma in situ .

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In vitro and in situ detection of melanocortin receptors in fibroblastic cell types of human skin

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Melanocortins such as alpha-melanocyte-stimulating hormone (alpha-MSH) or adrenocorticotropin (ACTH) elicit their pleiotropic effects by binding to specific surface receptors which are known as the melanocortin receptors (MC-Rs). In contrast to other cutaneous cell types expression and relevance of MC-Rs in fibroblastic cell types are poorly characterized. By means of RT-PCR analysis we show that expression of MC-1R in vitro is highly preserved in various human fibroblastic cell types of the skin. Neonatal and adult dermal fibroblasts, dermal papilla cells as well as HT1080 fibrosarcoma cells all express MC-1R at the RNA level. Expression of MC-1R at the protein level was confirmed in the above cell types by immunofluorescence studies and FACS analysis using an anti-MC-1R antibody directed against the amino acids 2-18 of the human MC-1R. Interestingly, MC-1R expression on the cell surface gradually declined during in vitro senescence of neonatal dermal fibroblasts. MC-1R expression in fibroblastic cell types in situ was confined to the dermal papilla and connective tissue sheath of the hair follicle while being undetectable within the interfollicular dermis. However, MC-1R expression was detectable by immune electron microscopy in dermal fibroblasts in situ. In light of the modulatory activities of alpha-MSH on collagen synthesis and deposition, these findings establish a basis upon which MC-1R and melanocortin expression can be investigated in fibrotic skin diseases. The preserved but distinct expression of MC-1R in various fibroblastic cell types, moreover, suggests additional functions of melancortins beyond collagen synthesis.

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Combined treatment of melanoma cells with telomere-homologue oligonucleotides (T-oligos) and 1,25 dihydroxy-Vitamin D3 (VitD3) or all trans-retinoic acid (ATRA) induces apoptosis more effectively than treatment with either of these agents alone.

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We have shown earlier that apoptosis of human melanoma cells can be achieved by treatment with either telomere-homologue oligonucleotides (T-oligos), 1,25 dihydroxy-Vitamin D3 (VitD3) or all trans-retinoic acid (ATRA). Here we investigated whether combination of the single substances would lead to an enhanced effect in vitro.

The highly metastatic human melanoma cell line G361 was incubated with either 20µM T-oligo, 10nM VitD3 or 1µM ATRA alone or with a combination of T-oligo/VitD3 or T-oligo/ATRA with the same concentrations.After 24 hours, cells were collected and processed for TUNEL-assay, subsequent FACS analysis and fluorescence microscopy.

Results revealed an induction of apoptosis by all the above treatments compared to diluent. The strongest induction, however, was seen in the cells treated with a combination of T-oligo/VitD3. In detail, while treatment with T-oligo led to an 1.7-fold increase compared to diluent treated cells, VitD3 and ATRA resulted in an 1.3-fold increase in apoptosis, respectively. The combined treatment with T-oligo/VitD3 led to an 11.9-fold increase and for T-oligo/ATRA a 9.4-fold increase compared to diluent. Thus, combining these treatments almost potentiates the pro-apoptotic effects.

While the nuclear receptors, gene regulatory elements as well as down-stream effector molecules that influence ATRA and VitD3 mediated cellular mechanisms have been well characterized, for T-oligos such data are still missing. It has been shown, however, that they do localize to the nucleus. All three agents share common downstream regulators, such as p21 and the E2F transcription factor, however they differ in their interaction with these.

Further studies are needed to fully elucidate the spectrum of actions for T-oligos, their interactions with VitD3 or ATRA and influence on possibly shared down-stream effector molecules. Understanding of these interactions might lead to new potential therapies for malignant melanoma.

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Freezing and thawing of dendritic cells for clinical immunotherapy: advantages using DMSO at a 5% concentration

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Dendritic cells (DC) are widely used for immunotherapy of cancer. Since the cells are often prepared as a "batch" and then stored in several aliquots for multiple immunizations of the patient, there is need to define the freezing, storage and thawing conditions providing the highest yield of viable and functional cells.
Here we describe the results of freeze-thaw experiments on DC obtained from several tumor patients. Dendritic cells were generated from monocytes with IL-4 and GM-CSF and matured using the standard cytokine cocktail (IL-1ß, IL-6, TNFa and PGE_2). Cells then were frozen in aliquots of 2 mio DC in freezing medium [10% HSA and 5% Glucose] with graded concentration (1,25; 2,5; 5; 10%) of DMSO. To mimick the clinical situation three different thawing conditions were compared: vials with cells in freezing medium were either left for 1 h at room temperature or 37ºC before washing the cells, or cells were thawed rapidly and directly washed without any storage. Viability of the cells was evaluated directly after washing via trypan blue staining (thawing rate). Cells were then seeded in aliquots of 0.5 mio cells in culture plates and survival determined after 24 and 65 h (wash out). For functional testing, the immunopotency of the cells was analyzed in a MLR with allogeneic T-cells (all with the same T-cell batch) and furthermore the migratory capacity studied in collagen gels using time-lapse videomicroscopy. In contrast to the most widely used storage of DC with 10% DMSO, cells frozen in 5% DMSO proved to be the most viable (thawing rate: 79,5 +/- 5,5 %; wash out 24h: 93,8 +/- 2,5% and 65h: 37 +/- 15%) and functionally equivalent to fresh non-frozen DCs regardless of the thawing conditions. Interestingly, DCs even did not loose their migratory capacity when analyzed for several hours in collagen gels containing 5% DMSO.
In summary our data 1) suggest that 5% of DMSO should be prefered for freezing of monocyte derived DC and 2) imply that thawed DC vaccine preparations containing up to 5% DMSO might be directly applied to patients without a washing step to remove DMSO.

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A basement membrane-like matrix formed by cell-released proteins at the medium/air interface supports growth of keratinocytes.

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Epithelial cells require adherence to a matrix for regular growth. During standard keratinocyte cell culture in serum-free medium, we observed that cell colonies formed not only on the bottom of the culture vessels but also at the medium/air interface. Coomassie blue staining detected a protein membrane that extended up to several centimeters between the colonies of floating cells. Ultrastructural investigation of this membrane revealed structures closely resembling those of basement membranes, and immunochemical staining confirmed the presence of laminins-1 and -5 as well as collagen IV, representative components of basement membranes. Cells attached to the floating membrane proliferated and could be cultivated for up to six months. When keratinocyte-conditioned medium was filtered and transferred to a culture vessel without cells, the protein membrane at the liquid/air interface formed within one week suggesting self-assembly of cell-released proteins. Our findings provide a basis for the production of epidermal basement membranes for potential medical uses.

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Lipid oxidation products induce expression of the anti inflammatory protein heme oxygenase -1 in keratinocytes and epidermal equivalent.

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A specific class of phospholipid oxidation products i.e. oxidized L-alpha-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholin (OxPAPC) that have been found in cell membranes of apoptotic cells, oxidized low-density lipoproteins, and at sites of inflammation, have recently been shown to directly act as potent inhibitors of acute, LPS-dependent inflammation. In addition, it was demonstrated that OxPAPC induces expression of Heme Oxygenase-1 (HO-1), an enzyme with potent anti-inflammatory properties, adding an unrelated mechanism by which oxidized phospholipids may influence inflammatory reactions.
When we studied the effects of lipid oxidation products on primary KC, HACAT and in an epidermal equivalent model, we found that OxPAPC induced expression of both HO-1 mRNA and protein. Addition of the radical scavanger bHT (butylated hydroxytoluene) did not inhibit the induction of HO-1 expression, indicating this process was not dependent on putative oxidative properties of OxPAPC. Since HO-1 has been implicated to play a role in wound healing by degrading free heme and thereby preventing free radical formation and lipid peroxidation, our data suggest that its induction by oxidized phospholipids represents a potential new therapeutic approach in the treatment of skin diseases associated with tissue damage mediated by oxidative stress.

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Effect of NO2 exposure on the expression of Der p 1 and Der p 10 in house dust mite (HDM) as assessed by RT-PCR

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Der p 1, a cystein peptidase, is one of the major allergens of the HDM Dermatophagoides pteronyssinus and causes allergic rhinitis or rhinoconjunctivitis in sensitized persons. Der p 10, tropomyosin, is a protein which should be expressed constitutively in the mites. We wondered whether the expression of these proteins would change under special conditions. For this purpose we established pure cultures of Dermatophagoides pteronyssinus species (obtained from Denmark and England) and repetitevly exposed culture aliquots to different concentrations of NO2 (50, 200, 400ppb). Subsequently we isolated the RNA of the various mites, transcribed it into cDNA followed by a PCR reaction specific for Der p 1 and Der p 10. Even at the single mite level Der p 1 and Der p 10 specific signal could be obtained. Interestingly, the danish mites expressed only low amount of Der p 1 under the influence of NO2 whereas the Der p 1 expression in the english mites only decreased at 400ppb NO2. On the other hand the danish mites expressed more Der p 10 in parallel to higher concentration of NO2. In this case the english mites showed less amounts of Der p 10 specific RNA. Factors like reproduction time or impaired survival may account for this. Taken together living conditions of HDM could have effects on the amount of allergen expression and be thus important for prevention measures with regard to HDM allergic individuals.

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Non-ablative skin rejuvenation with Erbium:YAG Laser pulses - investigation of structural changes in the skin -

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Objectives: Since several years ablative lasers like Er:YAG- and CO2-lasers has become a popular option for the treatment facial wrinkles. To evaluate the efficacy and safety of the Er:YAG laser used in a non ablative versus ablative manner in the treatment of facial rhytides, a multicenter study was initiated. The objective is to achieve selective, heat induced denaturalization of dermal collagen in solar elastosis that stimulates the production of new collagen with minimal epidermal injury.
Methods: Our clinical studies were conducted with a variable pulse Erbium:YAG laser (SubErb XL, WaveLight Laser Technologie AG, Erlangen, Germany) with defined repetitive thermal pulses: spot size 5 mm, fluence non-ablative pulses 3.1 J/cm², 3.5 J/cm² and 4.1 J/cm² respectively, fluence of the ablative pulses was 5 J/cm². Chances in epidermal and dermal structures were shown by optical coherence tomography (SkinDex, ISIS Optronics, Mannheim, Germany), histological sections and immunhistological staining prior laser treatment and 4 weeks after laser treatment. Furthermore transplanted human skin in an in vivo model was examined one, two and 4 days after laser treatment.
Results: Sub-ablative pulses as well as ablative pulses in Erbium:YAG laser treatment have an effect in the collagen metabolism in the upper dermis. Immunhistological staining demonstrates the expression of collagenase (MMP1) as well as the induction of pro-collagen synthesis in dermal fibroblasts even 4 days after treatment. Optical coherence tomography as well as histological sections show destruction of parts of the upper dermis followed by the production of new collagen bundles.
Conclusions: Non-ablative Erbium:YAG pulses are sufficient to induce structural chances in the upper dermis to obtain wrinkle reduction in sun damaged skin.

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Simple organotypic assay systems that imitate human hair follicle-like epithelial-mesenchymal interactions: Definition of basic criteria and suitable culture conditions

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Simplified organotypic systems are needed for dissecting the epithelial-mesenchymal interactions that underline human hair growth and as screening tools for candidate hair growth-modulatory agents. Here, we define basic criteria that such systems should meet and propose improved assay design and culture options, compared to previously published assays. Continuous submerged organotypic "sandwich" cultures were established. These consist of a pseudodermis (collagen I mixed with and contracted by human interfollicular dermal fibroblasts) on which one of two upper layers is placed: either a mixture of Matrigel? and follicular dermal papilla fibroblasts (DPC), with outer root sheath keratinocytes (ORSK) layered on the top ("layered" system), or a mixture of Matrigel™, DPC and ORSK ("mixed" system). Morphological and functional characteristics of these ?folliculoid sandwiches? were then assessed by quantitative immuno-histomorphometry. In both "layered" and ?mixed? systems, the ORSK formed spheroid epithelial cell aggregates which retained their characteristic keratin expression pattern (i.e. cytokeratin 6), while the DPC retained their characteristic expression of Versican. ORSK proliferated better in the "mixed" than in the "layered" sandwich system, regardless of the calcium or serum content of the media, whereas apoptosis of ORSK was lowest in the "mixed" system in serum-free, low calcium medium. The kinetics of DPC proliferation and apoptosis were similar in both systems. However, proliferation and apoptosis of DPC was higher in the presence of serum and/or under high calcium conditions. We propose a new organotypic submerged "folliculoid sandwich" system with serum-free, low calcium medium and a mixture of interacting human scalp hair follicle DPC and ORSK, which offers several advantages over previously available in vitro assays and reasonable approximation of epithelial-mesenchymal interactions in human scalp hair follicles.

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17-ß estradiol stimulates the growth of frontotemporal male scalp hair follicles *in vitro*

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While it is well-known that estrogens (17-ß estradiol, E2) can profoundly modulate hair growth in practically all mammals and that there is an inhibiting effect on hair shaft elongation, the effects of E2-administration on human scalp hair are still unclear. Recently, we have studied the effects of E2 on organ-cultured *female occipital* scalp hair follicles and confirmed the previously reported inhibition of hair shaft elongation. We now have investigated whether and how E2 alters the growth of *frontotemporal male* hair follicles *in vitro*.

To exclude other hormonal effects, anagen VI follicles from the frontotemporal scalp skin region from a single male individual were microdissected and organ-cultured in the absence of sebocytes over 9 days in serum-free William´s E medium, supplemented with L-glutamine, penicillin, streptomycin, insulin and hydrocortison, without and with E2 (1-100nM). Hair shaft elongation was measured on day 0,1,3,5,7 and 9. Surprisingly, hair shaft elongation was significantly stimulated by 1-100nM E2 during the whole culture period, starting already on the first day of culture (10nM, p=0.001). This correlated with a significant increase of proliferating-Ki67+ hair matrix cells on day 9 (10nM, p=0.05), there were no evident differences in apoptotic-TUNEL+ cells after 48 hours in culture, as well no alterations in the pigmentation of the hair follicle between E2- and vehicle-treated hair follicles. Compared to vehicle controls, the duration of anagen was slightly prolonged in the E2-treated hair follicles.These results raise the intriguing question whether there is a sex-and/or location-dependent difference of E2 effects in human scalp hair follicles. In addition it is crucial to further elucidate, to which extent the hair follicle response to E2 in a defined skin location depends on local differences in E2 metabolism (e.g. aromatase activity), estrogen receptor (ER) expression and E2-response of the key target genes of ER siginalling.

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More evidence for systemic oxidative stress in vitiligo

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Recently it was discovered that patients suffering with the depigmentation disorder vitiligo accumulate mM concentrations of hydrogen peroxide (H_2O_2) in their epidermis and µM levels in their blood (lymphocytes/monocytes). These unphysiological concentrations affect the structure and activities of a number of enzymes like catalase, glutathione peroxidase and 4a-carbinolamine dehydratase. The last enzyme is one of two in the recycling process of the essential cofactor 6R-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$) which is involved in the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, tryptophan and in the nitric oxide synthases (NOS). Dihydropteridine reductase (DHPR) is the second enzyme in this cofactor recycling process. Therefore we asked the question whether this enzyme was possibly also affected by H_2O_2. Here we assessed the level of oxidative stress of patients with vitiligo by measuring the DHPR activity in their whole blood. Initially it was shown that DHPR activity is strongly regulated by H_2O_2, with <30mM increasing the activity but with >30mM deactivating the enzyme. This effect is due to an oxidation of met 146 and met 151 in the active site sequence, consequently leading to disruption of the NADH-dependent binding site. This oxidation was confirmed by FT-Raman spectroscopy yielding the expected SO-band characteristic for methionine sulfoxide at 1025cm^{-1}. In addition, we demonstrated that DHPR activities of untreated vitiligo patients are significantly decreased as compared to healthy controls. Following removal of epidermal H_2O_2 with a topical pseudocatalase (PC-KUS), these enzyme activities are normalised. These new data provide more evidence for a systemic oxidative stress in patients with vitiligo. Therefore following DHPR activity could be a useful indicator of the oxidative status of these patients.

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The use of high-capacity (gutless) adenoviral vectors for the transduction of human dendritic cells (DC): Implications for immunotherapy using genetically modified DC

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Long-lasting, high-level gene expression in the absence of a toxic or inflammatory response to viral antigens is necessary for the successful application of genetically modified DC. We previously demonstrated that efficient transduction of mature DC using conventional ?E1?E3 adenoviruses (Ad) suppressed their stimulatory capacity for T cells. The current study is designed to investigate in more detail the characteristics of several, partly modified high-capacity (gutless) Ad-vectors that differ in viral gene expression from conventional ?E1?E3 adenovirus. All viral coding sequences are deleted from the vector genome. Thus, viral proteins cannot be expressed from the vector resulting in reduced toxicity and reduction of unexpected adverse events. We demonstrate that gutless Ad-vectors are suitable for an efficient transduction of mature human DC. DC transduced with gutless Ad-vectors do not exert any immunosuppressive effects but show a high allostimulatory capacity for CD4+ and CD8+ T cells when compared to DC transduced using conventional ?E1?E3 Ad-vectors. Thus, the suppressed stimulatory capacity for T cells of ?E1?E3 Ad-transduced mature DC seems to be the result of early adenoviral gene expression in DC that can be prevented using gutless Ad-vectors for transduction. The modification of gutless Ad-vectors by changing defined amino acids leads to a further amelioration of transduction of human DC. We can demonstrate an early and high transduction efficiency even at an MOI of 30 as well as a long-lasting gene expression for about one week after transduction. These results have important implications for the use of adenoviral vectors and/or genetically modified DC for therapeutic application.

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Stable transgene expression in keratinocytes (KC) transduced in-vivo by retroviral vector producing KC followed by topical selection for a selectable marker gene

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Naked DNA can be quite efficiently transfered into skin but this results only in short-term gene expression in keratinocytes (KC). In contrast, retroviral vectors (RV) are able to integrate into the genome, resulting in long-term gene expression. However, direct RV-delivery into skin is very inefficient. Another approach would be to transduce KC in-vivo by neighboring KC producing RV after DNA transfection. In this approach intradermal DNA-injection with all transacting genes necessary for RV formation will result in RV-producing KC. Topical selection for a selectable gene included in the RV would allow to further increase the number of transduced KC. To test feasibility human KC were transfected in-vitro with one plasmid containing all transacting RV genes (pPAM3) and another one harboring the multi drug resistance gene (pMDR) as a selectable gene. FACS analysis showed MDR+KC in about 1% of KC transfected with either pMDR or both pMDR and pPAM3. When treated with colchicine (50 ng/ml), pMDR-transfected KC stopped to proliferate while KC co-transfected with pMDR and pPAM3 continued to grow with 34-95% showing persistent MDR-expression. We then tested this model in a large animal model closely reflecting human skin. Pigs were intradermally injected with pPAM3, pMDR, or both (50 ug DNA) and skin biopsies taken at different time points for immunohistochemistry (IHC). In skin injected with either pMDR or both pMDR and pPAM3, about 10% of KC were MDR+KC but MDR-expression was lost over time. To perform selection on pig skin the optimal colchicine dose was determined by topical application of different dosages for 2 weeks. While 100-500 ug/g resulted in a dose-dependent increase of KC blocked in mitosis, higher doses (1000 ug/g) led to abundant KC necrosis. When treating DNA-injected skin with 500 ug/g for 2 weeks, in contrast to transfection with pPAM3 or pMDR, in co-transfected skin MDR+KC localized to hair follicles were seen. Thus, intradermal DNA-injection with transacting RV genes and a selectable gene followed by topical selection presents a promising approach to achieve persistent gene expression in the skin.

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Expression of two novel extracellular matrix proteins, matrilin-2 and matrilin-4, in human skin

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The family of matrilins are modular proteins belonging to a superfamily containing von Willebrand factor A-like domains. Four different matrilins have been identified and recent evidence suggests that they act as adaptor proteins connecting collagen fibrils to other components of the extracellular matrix. Matrilins are immunogenic and matrilin-1 (matn-1) has been shown to play a major role in the pathogenesis of relapsing polychondritis. To analyze the expression and function of matrilins in human skin, protein and mRNA distribution of matn-1, matn-2, matn-3, and matn-4 were studied. In addition, immunoblotting and RT-PCR were used to investigate the expression of matn-2 and matn-4 in keratinocyte and fibroblast cultures. In normal skin, matn-2 and matn-4 proteins were strongly expressed along the dermal-epidermal junction and around blood vessels by immunohistochemistry, whereas matn-1 and matn-3 were only faintly expressed at the basement membrane. Staining of salt-split skin demonstrated expression of both matn-2 and matn-4 at the lower, dermal part of the basement membrane. In accordance with the protein distribution, RT-PCR using RNA extracted from whole skin showed expression of matn-2 and matn-4 mRNA, but not matn-1 and matn-3 mRNA. Primary human fibroblasts and keratinocyte cultures were both found to express matn-2 and matn-4 protein using immunoblotting, however, marked differences in the mobility of the matn-2 and matn-4 bands were observed between fibroblasts and keratinocytes, presumably corresponding to post-translational modifications and proteolytic processing specific for each cellular origin. These findings demonstrate that matn-2 and matn-4 are expressed in skin, they are mainly deposited at the dermal side of the basement membrane and around blood vessels, and they may thus contribute to cutaneous homeostasis and dermal-epidermal adherence.

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The murine hair follicle is a melatonin target

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The prototypic pineal hormone, melatonin reputedly exerts many functional effects on mammalian skin and/or its isolated cell populations in culture (e.g., melanogenesis inhibition, melanocyte growth inhibition, regulation of seasonal changes in the pelage), and is recognized as a potent free radical scavenger. However, little is known about the cutaneous expression and regulation of melatonin *in situ*, and the functional role of melatonin in normal skin and hair follicle biology is still obscure. The aim of this study was to prove that murine and/or human hair follicles *in situ* are indeed peripheral melatonin targets and express melatonin membrane receptors (MT1, MT2) and/or the mediator of nuclear melatonin signaling, the orphan nuclear receptor RORa. Immunohistochemistry revealed that C57BL/6 mouse hair follicle keratinocytes *in situ* show prominent MT1-like immunoreactivity (IR), while it is only weak in murine dermal papilla fibroblasts. MT1-like IR in the hair follicle epithelium changed substantially in a hair cycle-dependent manner, suggesting a role of melatonin in murine hair cycle control. RORa-like IR was also detected in murine hair follicles, and also displayed hair cycle dependence (i.e., during early anagen RORa-like IR was strongly expressed in dermal papilla fibroblasts, while in late anagen and catagen it was maximal in the inner and outer root sheath). RT-PCR of MT1 and MT2, and real time PCR for RORa on C57BL/6 mice skin cDNA revealed that all three genes are transcribed in normal mouse skin, and demonstrated that their expression transcription is hair cycle-dependent. Functionally, melatonin (0.01 to 1 nM) significantly inhibited the constitution level of epidermal and hair follicle keratinocyte apoptosis in short term-mouse skin organ culture. In conclusion, we here provide evidence that normal murine skin is a prominent target for melatonin bioregulation, that it expresses MT1, MT2 and RORa and that at least some of these regulators are functionally active *in situ*.

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Treatment With The Angiogenesis Inhibitor Vasostatin Resulted In Decreased Delayed-Type Hypersensitivity Reactions In The Ear Skin Of Mice

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Recent data have demonstrated the importance of angiogenesis in the context of inflammation. Mice that overexpress placental growth factor (PlGF) sustained an increased cutaneous inflammation. Conversely, PlGF-deficient mice manifested a diminished inflammatory response while mice that lack the endogenous angiogenesis inhibitor thrombospondin 2 (TSP-2) exhibited a prolonged inflammation. Yet, little is known about the effect of antiangiogenic treatment on the extent of a cutaneous inflammation. Vasostatin is the 180 amino acid NH2-terminal domain of calreticulin that is known to inhibit angiogenesis in vitro and in vivo and has been shown to potently suppress tumor growth. To elucidate the effect of anti-angiogenesis therapy during inflammation, we induced delayed-type hypersensitivity (DTH) reactions in the ear skin of wildtype FVB mice with or without vasostatin treatment. Twenty-four and 48 hours after the initial challenge, the vasostatin-treated mice exhibited 38% and 31% less edema respectively when compared to the untreated mice, a trend observed throughout the investigated recovery period of 7 days. Furthermore, we found a significant decrease in blood vessel density in the inflamed ears of the vasostatin-treated mice. Vessel spread analysis combined with three-dimensional vessel imaging using whole mounts of ears following lectin perfusions demonstrated fewer larger vessels in the treated group compared to the control animals. Evans blue leakage assays revealed 37% less plasma leakage and intravital microscopy showed 30% fewer adherent and rolling leukocytes in the inflamed ears of vasostatin-treated mice, probably accounting for the decreased extent and duration of the inflammatory response. Our study revealed a novel effect of vasostatin on endothelial cell function and suggests that therapeutic approaches using angiogenesis inhibitors could prove beneficial in the treatment and prevention of cutaneous inflammation.

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Development and pharmacological manipulation of the nude (Foxn1 nu/nu)phenotype hair follicle

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Nude mice have a mutation in the transcription factor Foxn1, resulting in downregulation or complete loss of hair keratins 2-6. As a consequence, hair fibers do not develop normal cuticles and become structurally weak, curl within the follicular infundibulum and break off at the surface, resulting in alopecia. It is yet unknown which other abnormalites of hair follicle morphogenesis and/or cycling are associated with the absence of functional Foxn1. Cyclosporin A (CsA) can induce hair growth in nude mice, yet the effects of CsA on hair growth and cycling in nude mice are still very ill-defined. Therefore, back skin was harvested from hetero- and homozygous NMRI Foxn1/nu mice during different time points of postnatal skin development . Some NMRInu/nu mice were treated with CsA (30 mg/kg/d i.p.) or vehicle. Back skin was harvested after 7, 14 and 28 days and subjected to quantitative histomorphometry, immunohistochemistry and /or semi-quantitative RT-PCR . Hair follicle development in NMRI Foxn1nu/Foxn1nu mice appeared to be normal until morphogenesis stage 5, i.e. when the first hair shaft enters the infundibulum and starts to bend and coil. No other significant abnormalities in hair follicle morphogenesis, cycling, hair matrix keratinocyte proliferation or apoptosis were detected, compared to age-matched heterozygous mice.Therefore, in murine skin, FOXN1 indeed seems to be functionally important only in its recognized role in the control of hair keratin transcription. In telogen NMRInu/nu mice treated with CsA , anagen was induced prematurely, along with macroscopically visible hair shaft formation after 8 days. In CsA-treated anagen NMRInu/nu mice, hair growth was seen after 16 days, while vehicle controls showed only scattered hair growth after 18 days. Contrary to our previous findings in C57BL/6 mice, the CsA dosing regime used here failed to significantly modulate anagen duration/catagen induction. This implies that the hair growth-promotion by CsA in NMRI nude mice is primarily based on premature anagen induction. The study of CsA effects in nude mice offers a biologically and pharmacologically most intriguing, almost unexplored model for dissecting how a simple (immunosuppressive) drug can at least in part compensate for a functionally deficient transcription factor (FOXN1).

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Human hair follicles respond to corticotropin-releasing hormone (CRH) stimulation and display a cutaneous equivalent of the hypothalamic-pituitary (HP) axis

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Hair follicles operate as independent peripheral endocrine organs, which synthesize, metabolize and/or process many hormones, and the response to environmental stressors may affect this follicular endocrine system. It has been proposed that mammalian skin contains an equivalent of the HP-adrenal axis that regulates skin stress responses and that the hair follicle is an integral component of this skin stress system (SSS). We have reported that the murine hair follicle is a source and target of corticotropin releasing hormone (CRH), the most proximal element of the hypothalmic-pituitary-adrenal (HPA) axis and the major regulator of POMC gene expression and ACTH and a-MSH production. Here, we show by immunohistology and/or RT-PCR that human scalp hair follicles express all key components of the HP, in particular CRH, CRH receptor-1, POMC, a-MSH and ACTH. In order to explore the hair follicle response to CRH stimulation, microdissected, organ-cultured human scalp anagen VI hair follicles were treated with CRH. CRH-treated hair follicles showed a significant decline in hair shaft elongation and in hair matrix cell proliferation *in vitro*. Using a high sensitivity immunofluorescence technique (TSA), both ACTH and a-MSH immunoreactivity *in situ* were enhanced by CRH in selected compartments of the proximal hair follicle epithelium. By RT-PCR, POMC mRNA steady-state levels were significantly up-regulated in CRH-treated proximal hair follicles. This provides evidence that the human scalp hair follicle is both a source and a target of CRH, and displays a functional equivalent of the central HP axis. This may e.g. serve to integrate hair growth and pigmentation into cutaneous and systemic responses to environmental stressors, and may locally modulate the hair follicle immune system (HIS).

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Targeted Epidermal Thrombospondin 1 Overexpression Results In Decreased Inflammation And Vascular Remodeling During An Experimental Delayed-Type Hypersensitivity Reaction

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Thrombospondin 1 (TSP-1) is a potent endogenous angiogenesis inhibitor that is produced by several cell types including endothelial cells, keratinocytes and fibroblasts. In normal human skin, TSP-1 is deposited in the basement membrane of the dermal-epidermal junction and is thought to play an important role in maintaining vascular quiescence. However, TSP-1 has been detected in inflammatory diseases such as arthritis and has been described as pro-angiogenic and pro-inflammatory in some experimental models. In order to shed some light on the function of TSP-1 in a cutaneous inflammation, we studied the expression of TSP-1 in human allergic contact dermatitis (ACD). TSP-1 protein expression was potently upregulated in the inflamed lesions when compared to normal skin taken from the same patient. Moreover we found an almost 4-fold increase in the level of TSP-1 mRNA expression in inflamed lesions compared to normal skin. In-situ hybridization revealed that keratinocytes and endothelial cells were the main source of TSP-1 in ACD although scattered inflammatory cells were also positive. Computer-assisted morphometric image analysis of collagen IV-stained sections revealed vascular remodeling, demonstrated by an increase in the number of larger blood vessels, in the inflamed lesions when compared to uninflamed skin. To functionally characterize the role of TSP-1 in inflammation, we induced delayed-type hypersensitivity reactions in the skin of mice with targeted epidermal TSP-1 overexpression and found decreased edema formation in these mice when compared to wildtype littermates. We also found a significant decrease in the number of larger blood vessels in the inflamed ears of the transgenic mice when compared to FVB controls. Our data indicate that TSP-1 suppressed vascular remodeling during an ACD and thereby decreased the extent of the experimental cutaneous inflammation, probably by downmodulating the effects of pro-angiogenic factors such as vascular endothelial growth factor A (VEGF-A).

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Stress modulates peptidergic innervation and degranulates mast cells in skin

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Stress is said to induce itchiness of the skin, exacerbate inflammatory skin diseases and worsen wound healing. In this context, neuropeptides such as the stress mediator substance P are acknowledged since some time to play a role not only in sensation but also in inflammation and exert trophic functions. E.g. we were recently able to show, that stress or treatment of mice with substance P is associated with mast cell degranulation, increased cutaneous inflammation and increased apoptosis in the hair follicle. We were also able to show that associated with the cyclic growth of hair follicles the peptidergic cutaneous innervation is subject to livelong plasticity. However, local interactions between the nervous and immune systems, especially under perceived stress, have rarely been reported. Here we show for the first time, that 24 and 48hrs after sonic stress exposure the number of SP-immunoreactive nerve fibres in the back skin of C57BL/6 mice with all there hair follicles in the resting phase of the hair cycle (telogen), when innervation is low, increased significantly over non-stressed mice with the strongest increase after 24 hrs. Such substance P immunoreactive nerve fibres contacted mast cells more frequently, which became significant after 48 hrs. At the same time the percentage of degranulated mast cells increased significantly after 24 and 48 hrs with the strongest increase after 48 hrs when, and an increase in apoptotic cells became significant after 48 hrs. Thus, interaction between peptidergic nerve fibres and mast cells may lead to the release of histamine, chemokines and cytokines and ultimately alter cutaneous inflammation and trophic functions in the skin. Investigation of local interactions between the peripheral nervous system with the immune system thus appears a new and promising field of research in the analysis of cutaneous inflammatory disease and wound healing.

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Sexual hormones utilize complex mechanisms to modulate sebocyte differentiation

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Sexual hormones are important in the maintenance of human skin. Being the endocrine "brain" of the skin, the sebaceous gland is major hormone target. While androgens stimulate sebocyte proliferation, estrogens do not to affect sebaceous gland growth. Regarding differentiation, androgens are expected to stimulate and estrogens to suppress sebaceous lipid synthesis, while estrogens enhance sebaceous lipogenesis in aged skin. The latter results have been disputed by current *in vitro* data indicating no direct effect of sexual hormones on sebocyte differentiation. To elucidate these contradictions we have investigated possible pathways which may be used by sexual hormones to modulate sebocyte differentiation. It has been postulated that androgen induction of lipogenesis requires the concomitant presence of peroxisome proliferator-activated receptor (PPAR) ligands. We found that arachidonic acid (AA)-derived leukotriene B_4, a natural PPARa ligand, and linoleic acid (LA), a natural PPARd ligand, induce sebocyte enlargement, accumulation of lipid droplets in the cytoplasm, and nuclear fragmentation. The combined administration of testosterone and AA or LA slightly increased sebaceous lipogenesis. In contrast, synthetic PPAR ligands were unable to enhance sebaceous lipogenesis. AA significantly stimulated the production of prostaglandins (PG) E_2 and 15deoxy? ^{12,14}-PGJ_2, a proposed natural PPAR? ligand. PPAR? expression was downregulated by the phytoestrogen genistein. 17ß-estradiol has been shown to induce the metabolism of PGD_2 to 15deoxy? ^{12,14}-PGJ_2. 17ß-estradiol increased IGF-I synthesis (+28%) and down-regulated IGF-IR expression (-37 to -48%), whereas IGF-I significantly induced sebaceous lipogenesis. In conclusion, sexual hormones are not directly active but are likely to utilize complex mechanisms, including known pro-and antiinflammatory pathways, to modulate sebocyte differentiation.

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Control of androgen receptor expression in human keratinocytes and in a reconstituted human epidermis model with selective antisense oligonucleotides

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Association of locally increased androgen activity and skin disorders is obvious in acne and androgenetic alopecia in males. In addition, testosterone was unexpectedly found to perturb the epidermal barrier. Blockade of androgen action via androgen receptor (AR) antagonism accelerates wound healing in aged individuals. Androgen activity on skin can classically be inhibited by systemic administration of compounds, which have strong affinity for AR and antagonize androgen binding to AR molecules. In this study we applied a new technology to realize the same purpose: We tested the activity of antisense oligonucleotides against the AR in primary human foreskin keratinocytes, human non-foreskin keratinocytes from young (30 y) and older (60 y) female donors, and reconstituted human epidermis (SkinEthic model). Reconstituted human epidermis is similar to *in vivo* human epidermis and features a functional permeability barrier. To transfer the antisense oligonucleotides into human keratinocytes an optimum liposome-mediated transfection system with Poly-L-ornithine (12 µg/ml) over 4 h was used. The transfection efficiency was assessed using FITC-labeled $(ACTG)_5$ random oligonucleotides, which were localized in cytoplasmic structures of the keratinocytes. AR expression on the protein level was investigated by Western blotting. Transient transfection of foreskin keratinocytes with phosphorothioate antisense oligonucleotides (PTO) revealed a reduction of AR expression (25%) compared to native keratinocytes after 14 h recovery time. The AR knock down in epidermal keratinocytes of the compared women was stronger in the older, more differentiated keratinocytes. After 24 h, AR expression level have returned back to the level of non-transfected cells. The effect could be reestablished by repetition of transfection. PTO and 2'O-methylribosyl (MRO) antisense oligonucleotides decreased AR expression at levels varying between 46% and 70% in the air-lifted reconstituted human epidermis after 18 h recovery time. The successful inhibition of AR expression in human keratinocytes and reconstituted human epidermis is the first step to develop topically efficient compounds with oligonucleotides.

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New insights into the nerve end organ of human skin.

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Bearing the sensory nerve end organ, the skin establishes contact to our environment. So far, the analysis of the cutaneous nervous system was dependent on the use of tissue serial sections. Since such samples inherently allow visualisation of only a small part of the mainly horizontally oriented nervous system of the skin, we searched for possibilities enabling a more comprehensive view.Here we present a method based on the immuno-staining of dermal sheet preparations for subsequent analysis by electron microscopy and light - or laser scanning microscopy. We used antibodies against PgP9.5 and NCAM/CD56, both showing a regular network of fibres covering the entire superficial dermis. The bulk of free ending nerve fibers ramified within 25 µm of the dermo-epidermal junction, whereas below that only larger nerve bundles were present. Along the course of nerve fibers we observed NCAM/CD56+ protrusions with diameters ranging from 5 to 15 µm. We further characterized these protrusions demonstrating the ultrastructural features of terminal non-myelinating Schwann cells ensheating nerve fibres. Depending on the body region, we detected between 140 and over 300 individual terminal Schwann cells per mm^2 skin surface. In a double staining for NCAM/CD56 and vWF we analysed the topographical relationship of the nerve end organ to the blood vessels of the skin.In conclusion, this novel method allows for the first time a complex three-dimensional depiction of the cutaneous nervous system over several cm^2. Additionally, terminal Schwann cells can be studied in detail in situ for the first time. Further application of this method may provide new impetus in the investigation of the cutaneous nerve end organ under physiological and pathological conditions.

Influence of MC1R and GSTT1 and GSTM1 genotypes on UV sensitivity

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Variations in the melanocortin-1 receptor gene (MC1R) and in the glutathion-S transferase genes mu1 (GSTM1) and theta 1 (GSTT1) are thought to influence UV-sensitivity at different levels. MC1R is one of the major genes that determine skin pigmentation because the melanocortin-1 receptor regulates eumelanin synthesis. GSTT1 and GSTM1 are expressed in the skin and detoxify products of oxidative stress reactions occurring in response to UV-irradiation. The possible influence of interactions of genetic MC1R and GST variants have not been thoroughly investigated yet.

We determined the minimal erythema dose (MED) of ultraviolet B (UVB) irradiation as well as the immediate pigmentation dose (IPD) and the delayed pigmentation dose (DPD) of ultraviolet A1 (UVA1) irradiation in 93 healthy volunteers. Genetic variations of the coding region of the MC1R gene were determined by direct cycle sequencing. GSTM1 and GSTT1 null genotypes were analyzed by multiplex PCR.

Six frequent and six rare variants of the MC1R gene were detected (allele frequency > 6 % and < 1.5%, respectively). The R151C and R160W polymorphisms were associated with red hair, fair skin type and nevus count. R151C was also associated with a reported history of severe sunburns before the fifteenth birthday. The R151C and R160W polymorphisms were associated with DPD and IPD of UVA1, but this association was related to the association of these polymorphisms with red hair and fair skin. Independent associations were found between the V60L polymorphism and the MED of UVB ($p = 0.0145$), and between the V92M polymorphism and the DPD of UVA1 ($p = 0.0174$). In contrast to earlier investigations, there was no association between GSTT1 and M1 genotypes and UVB sensitivity. Lack of GSTT1 was associated with a higher IPD of UVA1. Our results confirm complex interactions between MC1R variants and UV sensitivity that are partially independent of phenotypic characteristics. There was no evidence for a significant interaction of MC1R with GST variants in UV sensitivity.