P001

PBMC of nickel allergic and non-allergic individuals display different apoptotic thresholds upon exposure to nickel and metal ions released by coins

B. Summer[1], K. Ghoreschi[1], S. Barnstorf[1], G. Roider[2], P. Thomas[1]

[1]Klinik und Poliklinik für Dermatologie und Allergologie, LMU München, 80337 München, Deutschland
[2]Institut für Rechtsmedizin, LMU München, 80337 München, Deutschland

Upon stimulation with metal ions antigen-specific T-cell proliferation in vitro can be observed in peripheral blood mononuclear cells (PBMC) of allergic individuals. On the other hand after inappropriate stimulation or by toxic effects, apoptosis may occur. In this study we assessed the apoptotic threshold of human PBMC upon exposure to nickel or the combination of nickel and copper in vitro. PBMC of 7 nickel-allergic patients (history and patch test positive) and 9 non-allergic controls were cultured for 72h in the presence of either medium, PHA, NiSO4 or a nickel/copper combination (Euro-Eluate). Apoptosis was detected by Annexin V staining followed by FACS-analysis. To further characterize and detect cells at an early stage of apoptosis, the percentage of Annexin V positive and propidium iodide negative cells was determined. In comparison to Ni-allergics, the PBMC of the control patients more easily underwent apoptosis after stimulation with PHA (19,3% vs 15,4%), NiSO4 10-5M (0,66% vs 0,4%) and 3 different euro eluate concentrations (1/100 17,7% vs 4,9%, 1/1000 12,2% vs 2,3%, 1/10000 6,5% vs 1,8%). After stimulation with NiSO4 10-4M the apoptosis rate was similar in controls and nickel allergic patients (0,9% vs 1,0%). Furthermore, interindividual differences were seen with a few blood donors - irrespective of allergic or non-allergic status - showing high apoptotic rates. However all except one individual were non-smokers and none had ongoing disease or systemic medication. Thus our data suggest, that PBMC of nickel allergics in general respond to PHA and the here tested metals with less cellular apoptosis than the PBMC of the controls. In addition, cell viability and apoptotic threshold seems to differ among the individual blood donors. The significance of these findings in relation to clinical intolerance reactions is under investigation.

P002

Inhibition of the IL-4/IL-13 receptor system in combination with allergen specific immunotherapy in a mouse model for allergic airway inflammation

C. Hahn[1], J. Meinhard[1], E. B. Bröcker[1], S. M. Grunewald[1]

[1]Klinik und Poliklinik für Haut- und Geschlechtskrankheiten, 97080 Würzburg, Deutschland

Introduction:IL-4 and IL-13 are considered as key regulators for the development of atopic disease and the inhibition of the IL-4/IL-13 system was shown to efficiently prevent the development of the allergic phenotype. This study addresses the therapeutic potential of an IL-4/IL-13 inhibitor on the basis of a mutated IL-4 variant in combination with allergen specific immunotherapy in allergic airway inflammation in mice.
Methods:BALB/c mice were weekly sensitized intranasally with Ovalbumin for a 4 week period. Subsequently, they were subjected to allergen specific immunotherapy, where Ovalbumin was applied intranasally with increasing doses from 1µg –1mg over a 3 week period. In addition to the immunotherapy, mice were treated with an IL-4/IL-13 inhibitor. OVA specific antibodies were measured by ELISA. In addition, bronchoalveolar lavages were performed and checked for airway eosinophilia and IL-5 levels.
Results: Intranasal OVA sensitisation resulted in persisting IgE synthesis. In addition increased IL-4 and IL-5 levels combined with an increased number of eosinophils could be detected in the BAL fluid. Allergen specific immunotherapy induced a significant reduction of OVA specific IgE synthesis, decreased IL-4 and IL-5 levels in the bronchoalveolar lavage fluid and abolished airway eosinophilia in comparison to untreated OVA sensitised animals. However, inhibition of the IL-4/IL-13 system together with the allergen specific immunotherapy did not lead to a further reduction of all measured allergic parameters.
Conclusion : Allergen specific immunotherapy could efficiently reverse the allergic phenotype in our mouse model. However, the anti allergic effect could not be increased further by inhibition of IL-4 and IL-13 simultaneously. This may be relevant for estimating the therapeutic potential of IL-4/IL-13 inhibitors.

P003

Stimulated release and production of basic FGF in human mast cells

A. Krajewski[1], S. Guhl[1], M. Artuc[1], T. Zuberbier[1]

[1]Dept. of Dermatology and Allergy, University Hospital Charité, 10117 Berlin, Deutschland

Mast cells have been associated with fibroblast growth in scars and poliposis nasi.Recently the capability of mast cell to produce FGF2 and to activate Fibroblasts has been described. In the current studie we have therefore investigated the production and release of FGF2 under the influence of several stimuli and pro-inflammarory cytokines in mast cells. Purified human mast cells obtained from cosmetic breast surgery were challenged for 24h with Ca-ionophore, anti-IgE, substance P (SP), IL4, TGFβ and IL6. Supernatants and cell lysates were analyzed for FGF2 content by ELISA technique.Results, expressed as mean percentage of unstimulated medium control (n=6), show no effect of Ca ionophore and anti-IgE whereas a dosedependent increase of cellular FGF2 was seen with SP (40% at 15µM), IL4 (140% at 0.5ng/ml), TGFβ (130% at 600ng/ml) and IL6 (110% at 20ng/ml). All stimuli showed no significant increase of FGF2 released into the supernatants except slightly with SP (15%).The data show that production of FGF2 in human mastcells can be induced by several proinflammatory cytokines as well as the neuropeptide SP whereas neither the IgE nor the ionophore induced degranulation of mast cells has an effect on the synthesis of FGF2. Thus in a pathophysiological setting proinflammatory IgE independent stimuli can induce the synthesis of FGF2 but additional yet unknown factors are required for its release.

P004

Natural rubber latex and insect venom allergens share IgE epitopes

V. Mahler[1], T. Fuchs[2], C. Gutgesell[2], D. Kraft[3], R. Valenta[3]

[1]Dept. of Dermatology, University of Erlangen-Nuremberg, D-91052 Erlangen
[2]Dept. of Dermatology, University of Gottingen, 37075 Gottingen, Deutschland
[3]Dept. of Pathophysiology, University of Vienna, 1090 Vienna, Oesterreich

Background: Recent epidemiological data have suggested a significant association between latex sensitization and positive skin prick test responses to aeroallergens, food allergens and to one or more insect venoms. IgE cross-reactivity between natural rubber latex allergens and allergens from plant-derived foods is well recognized on clinical and molecular grounds. However, no information is available regarding the presence of common IgE-binding components in latex and insect venoms. Aim of the study was to investigate whether there are cross-reactive IgE-binding structures in latex, bee and wasp venom and to study their nature.
Materials and Methods: Patients (n=15) with insect venom allergy and concomitant latex sensitization were identified by case history and positive CAP FEIA to latex and at least one hymenoptera venom. The IgE-binding components in bee and wasp venom as well as in latex milk and glove extracts were characterized by IgE-immunoblotting with the patients and controls sera. The presence of cross-reactive IgE-binding components in the venoms and latex extracts was studied by immunoblot inhibition experiments. The possible involvement of carbohydrate epitopes in the constitution of cross-reactive IgE epitopes was investigated by periodate treatment of the nitrocellulose-blottet extracts.
Results: Preincubation of the patients sera with latex extract prior to incubation with the nitrocellulose blotted insect venoms strongly inhibited IgE-binding to venom allergens of approximately 16, 39, 43 and 55-95 kDa in bee venom and of approximately 39 kDa in wasp venom. Vice versa, the patients serum IgE-binding to latex extracts could be totally inhibited by preincubation with the insect venoms indicating that the venoms were the primary sensitizing agents. The cross-reactive IgE epitopes were sensitive to periodate treatment.
Conclusion: Insect venoms share IgE epitopes with latex allergens. Periodate-sensitive cross-reactive IgE binding structures (carbohydrates) may be responsible for positive serological test results to natural latex extract in patients with insect venom allergy without clinically relevant latex sensitization.

P005

Differentiation between acute and chronic delayed type hypersensitivity reactions by radiolabeled RGD-peptides

M. Kneilling[1], B. Pichler[2], L. Hueltner[3], R. Mailhammer[3], H. Braumueller[1], K. Ghoreschi[1], T. Biedermann[1], R. Haubner[2], W. Weber[2], M. Roecken[4]

[1]Ludwig-Maximilians-Universität München, Klinik und Poliklinik für Dermatologie und Allergologie, 80337 München, Deutschland
[2]Technische Universität München, Nuklearmed. Klinik u. Poliklinik, D-81675 München
[3]GSF Forschungszentrum, Institut für Klin. Molekularbiologie u. Tumorgenetik, 81377 München, Deutschland
[4]Eberhard-Karls-Universität Tübingen, Hautklinik, D-72070 Tübingen

Many organ-specific autoimmune diseases are caused by delayed type hypersensitivity reactions (DTHR). Angiogenesis plays a major role in progression and exacerbation of DTHR such as contact hypersensitivity reactions (CHSR) or atopic dermatitis. In this study we investigated the role of avb3-integrin in acute-(A-CHSR) and chronic contact hypersensitivity reactions (C-CHSR) using a glycosylated RGD-peptide with selective binding to the avb3-integrin. C57BL/6 mice were sensitized with 5% trinitrochlorobenzene (TNCB) on the abdomen. After 7 days animals were challenged at the right ear with 1% TNCB to elicit hapten specific A-CHSR. To induce C-CHSR animals were re-challenged at the right ear with 1% TNCB eight times. To investigate antigen independent chronic inflammation (AICI) animals were treated five times with phorbol ester (PMA) at the right ear. CHSR and AICI was determined by measuring ear thickness before and 12h after the final TNCB / PMA challenge. A total of 10 animals were i.v. injected with 10 uCi [125I]Gluco-RGD, and tracer uptake was determined 3h p. i. by autoradiography. Additionaly, 2 animals with A-CHSR and C-CHSR were injected with 250 uCi [18F]Galacto-RGD peptide and scanned in vivo with the small animal positron emission tomograph MADPET 1h p. i..The [125I]Gluco-RGD uptake ratio -right ear (treated) versus left ear (untreated)- was 2.9 for C-CHSR and only 1.3 for A-CHSR and AICI. In vivo PET images showed intense [18F]Galacto-RGD uptake in C-CHSR but not in A-CHSR. Average ear swelling responses were 120 um in A-CHSR (increase of ear thickness from 160 to 280 um), 240 um in C-CHSR (increase from 240 to 480 um) and 160 um in AICI (increase from 180 to 340 um). Immunohistochemical staining for b3 confirmed b3 expression on blood vessels in ears from animals with C-CHSR but but not with A-CHSR. avb3-integrin expression distinguished between acute and chronic DTHR. Radiolabeld RGD-peptides open new possibilities to examine angiogenesis.

P006

Nerve growth factor and its receptor TrkA are expressed in the human pilosebaceous apparatus, and modulate catagen development of organ-cultured anagen scalp hair follicles

M. G. Hansen[1], R. Overall[2,1], M. Schroeder[2,1], P. Pertile[3], P. C. Arck[2], R. Paus[1], E. M. Peters[2,1]

[1]University Hospital Eppendorf, University of Hamburg, Department of Dermatology, 20246 Hamburg, Deutschland
[2]Biomedical Research Center, Charité, Humboldt University, Psychoneuroimmunology, 13353 Berlin, Deutschland
[3]Cutech Srl., V-3649 Venice, Italy

Nerve growth factor (NGF) and other members of the neurotrophin-family of neuronal growth factors exert numerous functions in non-neuronal tissues. Since we have recently described a major role for neurotrophins in the control of murine hair follicle morphogenesis and cycling, we have now investigated whether NGF and its high-affinity receptor (TrkA) are also involved in human hair growth control. We found that both NGF and TrkA are prominently expressed in human scalp skin and isolated anagen hair follicles by rtPCR. NGF in situ labeling and immunoreactivity are dominant in more differentiated keratinocytes, such as the distal layers of the epidermis and the inner root sheath, but is also found in the hair matrix, dermal papilla cells, the arrector pili muscle and the sebaceous gland. Extrafollicular NGF-immunoreactivity is observed in epidermal Langerhans cells, dermal and subcutaneous mast cells and nerve fiber bundles. TrkA in situ labeling and immunoreactivity are dominant in the more undifferentiated keratinocytes of the basal epidermis and the outer root sheath with little expression is found outside the follicular compartment. These expression patterns of NGF and TrkA suggest para- and autocrine functions for TrkA-mediated signaling in epithelial growth control in the anagen hair follicle. In organ-cultured human scalp hair follicles high concentrations of NGF (50 ng/ml) decrease hair shaft elongation and prematurely induced catagen-like hair cycle progression. Taken together these data suggest that neurotrophins are also involved in human hair gowth control, and that NGF specifically interferes with tissue remodeling during the anagen-catagen transformation. TrkA ligands thus deserve to be explored as hair growth-modulatory agents in the management of hair growth disorders.

P007

DETECTION OF $CD123^+CD45RA^+CD4^+$ PLASMACYTOID DENDRITIC CELLS IN ALLERGIC CONTACT DERMATITIS

C. Bangert[1], G. Stingl[1], T. Kopp[1]

[1]University of Vienna Medical School, Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, A-1090 Wien,

To account for the growing complexity and diversity of the dendritic cell (DC) family, we searched for the presence of various DC subpopulations in both healthy and diseased skin. For this purpose we analyzed, by immunohistology, biopsies from positive 72h epicutaneous patch tests (EPTs; n=10) as well as from normal human skin (n=5). Normal human skin (NHS) contained $CD1a^+$ and $CD1c^+$ cells in both the epidermal (19.3/mm- and 4.3/mm basement membrane, respectively) and dermal (16.2/mm^2 and 28.6/mm^2, respectively) compartment. In EPTs the number of dermal $CD1a^+$ cells (47.5/mm^2) was significantly increased at the expense of their epidermal counterpart (12.6/mm basement membrane). A similar increase in number was observed in the $CD1c^+$ population of EPT specimens (15.7 epidermal $CD1c^+$ cells/mm basement membrane, and 100.8 dermal $CD1c^+$ cells/mm^2). As opposed to the situation in NHS, a substantial proportion of epidermal (28%) and dermal (38%) $CD1a^+$ cells in EPT lesions expressed the activation marker CD83, whereas. the majority of the CD1c+ cells in EPTs remained CD83-. These findings are indicative of an enhanced migration but distinct maturation of CD1a+ Langerhans cells (LCs) and $CD1c^+$ dermal DCs (DDCs) in hapten-challenged skin. We also detected a small number of $CD123^+CD45RA^+$ cells in the dermal, but not in the epidermal compartment of NHS (2.9/mm^2). Interestingly, these cells represented a substantial proportion of the entire DC infiltrate in EPT lesions. They were numerically increased already in 6h EPTs and peaked in 72h reactions (3.1 epidermal cells/mm basement membrane, and 47 dermal cells/mm2). By flow cytometry, these cells exhibited the $CD123^+CD45RA^+CD4^+$MHC II^+ phenotype described for plasmacytoid DCs (pDCs). In summary we find that, in contrast to NHS, EPT lesions not only contain increased numbers of LCs and DDCs, but also a significant population of pDCs. It remains to be determined whether the attraction of pDCs to the site of hapten challenge contributes to the evolution or resolution of allergic contact dermatitis.

P008

Correlation between interferon-gamma and inhibin B in seminal plasma of men

U. Hipler[1], G. Schreiber[1]

[1]Klinik für Dermatologie und dermatologische Allergologie, D-07740 Jena,

Objectives :The role of the cell-mediated immunity in male infertility is still far from clear. The function of interferon-gamma, a secretory product of activated T cells and natural killer cells, is unclear. Interferon-gamma exerts a variety of biologic effects such as the inhibition
of cell growth and the activation of the immune system. In normal individuals, the spleen, kidney, liver, peripheral blood leukocytes and placenta all produce a low level of interferon, which has physiologic roles. In contrast, interferons are associated with disease such as the viral infection. The expression of interferon-gamma and interferon-alpha in the rat testis has been reported . Therefore, we measured the seminal plasma level of interferon-gamma in donors and patients.
Material and Methods: The presence of interferon-gamma was investigated in seminal plasma
of 285 patients and 58 donors as control, using a specific enzyme-linked immunosorbent assay, in order to study its role in male infertility (ELISA by Bender MedSystemsTM, Wien, Austra, distribution by Biozol,Germany). Statistical analysis was performed using analysis of variance (ANOVA).
Results: Interferon-gamma was present in similar levels in the seminal plasma of patients (2.55 ±8.27 pg/mL) and of the control group (2.22 ±4.35 pg/mL).There was no significant difference between the group of patients and the control group (p= 0.769). Moreover, the correlation between interferon-gamma and spermcount, polymorphonuclear granulocyte elastase and inhibin B was evaluated. No significant correlations were observed between the levels of interferon-gamma in seminal plasma and the serum level of inhibin B.
Conclusions: In this study, the levels of interferon-gamma in the seminal plasma of patients and donors have been measured. Recently, the expression of IFN-alpha and gamma were reported in the rat testis. Peritubular and Sertoli cells are able to produce IFN-alpha protein only after viral stimulation. IFN-alpha1 mRNA is expressed in meiotic pachytene spermatocytes in early spermatids and Sertoli cells. In contrast, IFN-gamma protein was consistently detected in PHA stimulated early spermatid-conditioned medium, but not in non-stimulated medium. IFN-gamma mRNA was also found in early spermatids. At present, the function of IFNs in the testis and in the seminal plasma cannot be completely understood.

P009

P-1- 02 Epitestosterone - a new prospective anti androgen?

U. Hipler[1], G. Schreiber[1], J. Berlau[1], W. Roemer[2]

[1]Klinik für Dermatologie und dermatologische Allergologie, 07740 Jena, Deutschland
[2]Jenapharm GmbH & Co.KG, 07743 Jena, Deutschland

Objectives :The effect of epitestosterone on inhibin secretion by tat Sertoli cells in the presence and absence of folliclestimulating hormone (FSH) was studied.
Material and methods: Sertoli cells were isolated from testes of 18 day old Wistar rats by collagenase, DNase and hyaluronidase digestion and subsequent osmotic shock treatment with Tris/HCI on day 2 of culture. Contamination by gerin and peritubular cells was examined by nuclear morphology and Oil Red 0 staining and 2 or 3/o of contaminating cells were observed, respectively. On day 4 of culture, secreted inhibin in serum free medium was measured by ELISA, using Sertoli cells cultivated in the presence and absence of FSH and epitestosterone.
Results : FSH (300 ng/ ml) stimulated the secreted inhibin level by 2-3 fold. A 2- fold increased secretion of inhibin was observed in the presence of epitestosterone and absence of FSH but not in the presence of both.
Conlusions : It is concluded that FSH and epitestosterone may have similar stimulatory effects on inhibin secretion by Sertoli cells from immature rats in vitro. Furthermore, epitestosterone may negatively affect endocrine stimulation of spermatogenesis through its ability to increase the inhibin level directly and thereby indirecdy suppress the secretion of FSH.

P010

Micromanipulation of single cells from tissue imprints is an alternative to laser assisted microdissection

T. C. Brauns[1], M. Goos[1]

[1]Universitätsklinik Essen, Hautklinik, 45122 Essen, Deutschland

Background:
The investigation of single cells by modern molecular-biological methods like the PCR is of increasing interest in many different fields of research. Different techniques have been developed to obtain single cells from solid tissue. Currently the most frequently used methods are laser assisted microdissection techniques like the laser capture microdissection [LCP] and the PALM-technique (3D positioning and ablation with the laser microscope). But even laser assisted microdissection from tissue sections cannot exclude contamination of the targeted cells by underlying cell-fragments. Moreover these techniques can only be performed using an expensive Laser Microscope.

Methods:
We describe a method to obtain single cells from immunhistochemically stained tissue imprints and blood smears using a 27 gauge needle guided by a micromanipulator. Cells obtained were used in a single cell PCR targeting the gamma-chain of the T-cell receptor. Direct sequencing of the PCR-products was performed.

Results:
Using this method clonality of single atypical lymphocytes derived from a patient suffering from tumor stage mycosis fungoides could be demonstrated by direct sequencing.

P011

Elevated expression of EMMPRIN (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers

T. Hildenbrand[1], M. Idzko[2], E. Panther[1], E. Bandemir[1], M. Hartmann[3], J. Norgauer[1], Y. Herouy[1]

[1]University Hospital, Department of Dermatology, D-79104 Freiburg
[2]University Medical Center, Dept. of Pneumology, D-79106 Freiburg
[3]Dermatologisch-phlebologische Gemeinschaftspraxis, Hartmann & Waldermann, D-79104 Freiburg

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

P012

The vitamin D receptor is essential for normal skin function

S. Meindl[1], A. Rot[2], S. Chang-Rodriguez[1], W. Hötzenecker[1], S. Kato[3], H. Cross[4], A. Elbe-Bürger[1]

[1]VIRCC, DIAID, Dept. of Dermatology, 1235 Wien, Oesterreich
[2]Novartis Research Institute, 1235 Wien, Oesterreich
[3]Univ. of Tokyo, Institute of Molecular and Cellular Biosciences, 113 Tokyo, Japan
[4]Univ. of Vienna, Dept. of Pathophysiology, 1090 Wien, Oesterreich

1,25alpha-dihydroxyvitamin D3 [1,25(OH)2D3] the active metabolite of vitamin D, exerts its activities by binding to the vitamin D receptor (VDR) with subsequent function as a transcription factor. Targeted ablation of the VDR (VDR/KO) in mice results in rickets and alopecia. We used these mice to further study the function of VDR in skin physiology and specifically its role in Langerhans cell (LC) and dendritic epidermal T cell (DETC) biology. Epidermal sheets and freshly prepared epidermal cell suspensions from VDR/KO mice contained similar numbers of LC but increased densities of DETC compared to VDR wild type (WT) controls. Pretreatment of epidermal cells with 1,25(OH)2D3 resulted in a concentration-dependent inhibition of WT-LC but not VDR/KO-LC-driven lymphocyte proliferation. Trichrome staining of the skin of VDR/KO mice at the age of 4 months revealed deposition of collagen in the dermis. This was even more pronounced in older mice. In addition, sebaceous glands in the skin of VDR/KO mice were significantly enlarged. Our data show that (i) the VDR has no major influence on LC distribution, morphology and numbers, (ii) active hair growth is necessary to maintain the homeostasis of DETC, (iii) 1,25(OH)2D3 inhibits function of WT- but not VDR/KO-LC, and (iv) VDR expression may control collagen production.

P013

Phenotypic switch of endothelial cells in skin biopsies of different autoimmune diseases

C. Bester[1], T. C. Fischer[2], F. Serowka[1], P. Welker[1], D. A. Groneberg[3]

[1]Charite, Institute of Anatomy, 10115 Berlin, Deutschland
[2]Charite, Department of Dermatology and Allergology, D-10115 Berlin
[3]Charite, Department of Pediatric Pneumology and Immunology, 13344 Berlin, Deutschland

In cutaneous autoimmune diseases, the vascular endothelium is among the key targets for circulating mediators of inflammation and controls the trafficking of inflammatory cells and mediators from the systemic circulation toward the inflamed area. During angiogenesis and vascular remodeling, endothelial cells may change their phenotypus and express surface molecules which interact with circulating mediators such as vasoactive intestinal polypeptide (VIP). Here we examined the expression of the inducible typ 2 receptor of VIP, the most potent endogenous vasodilating peptide in cutaneous endothelial cells of autoimmune disease skin biopsies using immunochemistry.
In normal control skin tissues, endothelial cells do not express VIP type 2 receptor protein. In contrast, endothelial cells may undergo a phenotypic switch and express the receptor protein. In this respect, the endothelium of affected areas in bullous pemphigoid and morphea displayed receptor expression. This expression correlated to endothelial CD31 expression. In contrast, endothelial cell of chronic discoid lupus erythematodes did not exhibit type 2 receptor expression.
The present studies demonstrate a differential expression of the inducible VIP receptor in cutaneous endothelial cells of different autoimmune diseases. A phenotypic switch of the endothelium may therefore be induced by disease-specific factors rather than by general inflammatory stimuli.

P014

Expression of the vasoactive intestinal polypeptide receptor VPAC2 in cutaneous mastocytosis

A. Kretschmer[1], D. A. Groneberg[2], F. Serowka[1], T. C. Fischer[3], P. Welker[1]

[1]Charite, Institute of Anatomy, 10115 Berlin, Deutschland
[2]Charite, Department of Pediatric Pneumology and Immunology, 13344 Berlin, Deutschland
[3]Charite, Department of Dermatology and Allergology, 10115 Berlin, Deutschland

Vasoactive intestinal polypeptide (VIP) is a modulatory peptide which has been demonstrated to inhibit degranulation and changes in the granular content of mast cells. As these effects of VIP are dependent on the expression of its receptors on mast cells, the present study assessed the expression and regulation of the inducible VIP receptor VPAC2 in normal human skin and skin disorders related to increased numbers of mast cells. To identify the protein and mRNA expression of VPAC2, RT-PCR and immunohistochemistry were carried out.
RT-PCR demonstrated the expression of VPAC2 in isolated, highly purified human mast cells. Serial-section immunohistochemistry for mast cell-specific tryptase and chymase and VPAC2 confirmed the transcriptional data and showed a colocalisation of the mast cell markers and the VIP receptor. Immunohistochemistry for VPAC2 and tryptase in skin biopsies in mastocytoma demonstrated that all mast cells in the tumour infiltrate expressed the receptor. The staining-intensity was decreased in comparison to normal control skin.
In sections of urticaria pigmentosa biopsies, marked differences in cellular staining intensity were found for affected and unaffected areas: A decrease of intensity from normal control skin to unaffected areas to affected areas was found.
In conclusion, the present data demonstrate the expression of a VIP receptor in human skin mast cells. Its decrease in cellular expression in mast-cell related skin disorders may reflect a pathophysiological role of VIP in different forms of mastocytosis.

P015

Localized scleroderma is associated with activation of antioxidant enzymes and increased lipid peroxidation

C. S. Sander[1], S. Kleemann[1], P. Elsner[1], J. J. Thiele[1]

[1]Friedrich-Schiller-University, Department of Dermatology, D-07743 Jena

Localized scleroderma (morphaea) is a connective tissue disease characterized by fibrosis of circumscribed skin lesions. Although the origin is still unknown, it is postulated that the disease process involves damage to the vasculature, immune activation and connective tissue dysregulation. Oxidative stress is discussed as one of the major contributors, but convincing in vivo evidence is still lacking. We hypothesized that the natural redox balance is disturbed and results in accumulation of lipid peroxidation products (LPO) in scleroderma lesions. LPO products, such as malondialdyde (MDA) or 4-hydroxynonenal (4-HNE) tie spontaneously to DNA, RNA or proteins and thus lead to oxidative tissue damage.
Biopsies of patients with localized scleroderma (n=16) were compared to healthy skin of age-matched patients with benign melanocytic naevi (n=16). Expression of the antioxidant enzymes copper-zinc superoxide dismutase (CuZnSOD), manganese SOD (MnSOD) and catalase (CAT) was analyzed by immunohistochemical techniques. To detect lipid peroxidation products, protein-bound malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were investigated. In all cases, stained sections were analyzed using densitometric image analysis (Analysis 3.0, Soft Imaging System, Muenster, Germany). Statistical analysis was performed by ANOVA.
In human scleroderma biopsies, a significant overexpression of the antioxidant enzyme catalase was found within the epidermis and dermis when compared to age- and site-matched control tissue. Intriguingly, the lipid peroxidation markers MDA and 4-HNE were significantly increased in scleroderma tissue. LPO products were found not only in epidermal keratinocytes cells, but also occurred in dermal collagen tissue.
The activation of enzymatic antioxidants as well as the accumulation of lipid peroxidation products found in localized scleroderma are indicative of oxidative damage. Thus, oxidative stress is very likely to play a pathophysiological role in the progression of localized scleroderma.

P016

Tissue counter analysis as a tool for the discrimination of skin tumors: A survey of the method.

M. Wiltgen[1], A. Gerger[2], J. Smolle[2]

[1]Institut für Medizinische Informatik, Statistik und Dokumentation, A-8036 Graz
[2]Univ.-Klinik für Dermatologie, Analytisch-morphol. Dermatologie, A-8036 Graz

Microscopic views of histological tissues show structures arranged in a variety of patterns. Therefore the automatic segmentation of different structures is difficult, and cannot be done in a general approach. Nevertheless, automatic discrimination of tissue structures plays an important task in medical image analysis due to an increasing need of objectivity and assessment in diagnosis.
In this study we present tissue counter analysis (TCA) as a method for discrimination of different histological tissues. TCA is based on the partition of the image into square elements of equal size where the features, describing the tissue, are calculated out of each square element. Because TCA needs no image segmentation, problems related to this task are avoided.
In this study we test the discriminative power of grey level histogram features and of co-occurence matrix features on the discriminative power in benign common nevi and malignant melanoma lesions. 80 cases from microscopic views of benign common nevi and malignant melanoma were sampled. From this set 40 cases were randomly selected as learning set and the remaining 40 cases were used as test set. The classification was done by CART (Classification and Regression Trees) analysis. The classification correctly classified 94,7 % of nevi elements and 92,6% of melanoma elements in the learning set. In the test set, discriminant analysis based on the percentage of “malignant elements” showed a correct classification of 100% of the cases of benign common nevi and 95% of the cases of malignant melanoma. The classification results were indicated in the original image in order to evaluate the performance of the procedure and to compare the elements considered to be diagnostic by the automated procedure with the assessment of routine diagnostic histopathology.
In conclusion, tissue counter analysis is a potential diagnostic tool in automatic or semi automatic analysis of melanocytic skin tumors and facilitates the recognition of diagnostically relevant morphologic areas.

P017

Inhibition of IFN-γ-Induced Keratin 17 Overexpression as a Putative Psoriasis Autoantigen by Antisense Oligonucleotides *In Vitro*

R. Boeckelmann[1], T. Horn[2], H. Gollnick[1], B. Bonnekoh[1]

[1]Otto-von-Guericke-Universität Magdeburg, Klinik für Dermatologie und Venerologie, 39120 Magdeburg, Deutschland
[2]Otto-von-Guericke-University Magdeburg, Institute of Medical Neurobiology, 39120 Magdeburg, Deutschland

Psoriasis is a common, chronic-relapsing inflammatory skin disease with a not yet fully understood etiopathogenesis, resembling an autoimmune disease driven by T-cells. Recently an epitope of keratin 17 (K17) has been described as a putative major target for autoreactive T-cells in psoriasis [Clin Exp Immunol 117:580, 1999]. With respect to the so-called IFN-γ/K17 autoimmune loop concept [Skin Pharmacol Appl Skin Physiol 14: 217, 2001], we studied the potential of antisense phosphorothioate oligodesoxynucleotides (ODN) to inhibit the expression of such presumptive autoantigens *in vitro*.
One day after plating, subconfluent HaCaT keratinocytes were incubated with IFN-γ and ODN of various sequences complementarily directed to K17 mRNA (Biognostik). FITC- labeled and ?unlabeled non-sense ODN served as appropriate controls. K17 expression was measured by immunoflow-cytometry after 3 days of incubartion.
Confocal laser scanning microscopy proved the uptake of FITC-ODN by HaCaT keratinocytes to reach a maximum plateu level at 24 hrs. The expression of K17 was significantly increased by IFN-γ up to 588±142% as compared to 100% of the untreated control. This effect was found to be significantly inhibited by a given antisense ODN down to 374±91% (n=7 independent experiments, $p<0.05$).
Thus, the expression of K17 was successfully inhibited by antisense ODN in vitro, which could open future avenues to gene therapeutic approaches for the treatment of psoriasis.

P018

Molecular Genetic Analysis Excludes Implantation Metastasis of Opposite Basal Cell Carcinomas

C. Hafner[1], A. Hartmann[2], R. Knuechel[2], W. Dietmaier[2], M. Landthaler[1], T. Vogt[1]

[1]Universitaet Regensburg, Klinik und Poliklinik fuer Dermatologie, 93042 Regensburg, Deutschland
[2]Universitaet Regensburg, Institut fuer Pathologie, 93042 Regensburg, Deutschland

Basal cell carcinoma (BCC) of the skin is the most common tumor in the white population. BCCs are characterized by invasive growth, but usually do not metastasize. Herein we describe a 66-year old patient who developed two opposite BCCs, a large ulcerated one at the left parietal region of the head and a small one at the helix of the left ear. The two lesions matched exactly when pressing the ear against the head, suggesting an implantation metastasis mechanism, which has neither been published nor further analysed with regard to tumor clonality. An implantation mechanism would challenge our current view of the nature of BCCs.
Therefore, molecular genetic techniques were used to confirm or exclude such a mechanism in this rare clinical constellation. Tumor tissue was precisely microdissected for DNA isolation. Exons 5-9 of the p53 tumor suppressor gene were directly sequenced. In addition, loss of heterozygosity analysis of chromosome 9q was performed using five polymorphic microsatellite markers. The BCC of the ear revealed a p53 mutation at codon 273, whereas the other one lacked this mutation. In addition, the smaller BCC showed loss of heterozygosity at 9q33.3, in contrast to the larger BCC. Interestingly, histologically normal skin of the ear distant from the small BCC had the same deletion, indicating a field defect of this skin patch.
We conclude that molecular genetic analysis of this rare case clearly demonstrated a different origin of the two BCCs and therefore could exclude a mechanism of implantation metastasis.

P019

Angiotensin AT1- and AT2-receptors are upregulated in human cutaneous wounds

U. M. Steckelings[1], B. M. Henz[1], S. Wiehstutz[1], M. Artuc[1]

[1]Humboldt Universitaet - Charite, Dermatologie, 10117 Berlin, Deutschland

We recently showed the expression of angiotensin AT1- and AT2-receptors on diverse human primary cutaneous cells in vitro as well as in the epidermal layer of skin sections ex vivo. In order to elucidate, whether the expression of AT-1 or AT2-receptors is altered in cutaneous wound healing, we performed two sets of experiments:
Firstly, we used the common model of artificially wounding of cultured human primary keratinocytes by razor blade scraping. Receptor expression was determined by semiquantitative RT-PCR (normalised against GAPDH expression) 1, 3 and 12 hours after scraping and compared to that on non-manipulated cells. Secondly, skin biopsies were kept in organ culture, cut by scalpel to a defined depth, and receptor expression examined by immunohistochemistry using polyclonal antibodies directed against the human AT1- or AT2-receptor, respectively.
Artificial wounding of keratinocytes resulted in a marked upregulation of both receptor subtypes, but with a different time-course: while AT1-receptors were maximally upregulated already 1 hour after scraping and returned to baseline levels after 3 hours, AT-2 receptor upregulation peaked not earlier than 3 hours after wounding and returned to baseline levels only after 12 hours.
Skin biopsies were forwarded to immunohistochemistry 24, 48 and 72 hours after cutting. After 48 hours, the cut was almost closed by a newly formed keratinocyte layer, and a new keratinocyte layer was also grown out from the edges of the biopsies. Expression of both, AT1- and AT2-receptors, was markedly upregulated within the newly formed epidermal layers.
From our results, we conclude, that the expression of angiotensin AT1- and AT2-receptors is upregulated in cutaneous wounds, indicating that the renin-angiotensin-system may be involved in wound healing mechanisms in human skin.

P020

Reactivity to autologous serum skin test, clinical features, and anti-$Fc_\varepsilon RI$/anti-IgE expression in patients with chronic urticaria

P. Staubach[1], K. Onnen[1], A. Kromminga[2], A. Hanau[1], M. Metz[1], M. Magerl[1], I. Tschentscher[3], J. Knop[1], M. Maurer[1]

[1]University Hospital Mainz, Department of Dermatology, 55101 Mainz, Deutschland
[2]Institute for Immunology, Clinical Pathology, Molecular Medicine, 22339 Hamburg, Deutschland
[3]Koordinationszentrum für klinische Studien, 55131 Mainz, Deutschland

Recent studies have called into question the importance of histamine releasing autoantibodies (anti-$Fc_\varepsilon RI$ or IgE anti-IgE) in the pathogenesis of autoimmune chronic urticaria (CU), characterized by urticarial skin reactions after intradermal challenge with autologous serum (= autologous serum skin test, ASST). Here, we have characterized the clinical profile of this hard to treat subgroup of CU patients, and we have assessed the presence of anti-$Fc_\varepsilon RI$ and anti-IgE in ASST+ and ASST- CU patients. We found positive ASST reactions in 53 of 152 CU patients (=35%) and analysed 1) duration and severity of CU (i.e. numbers, size and/or duration of the CU symptoms weals, pruritus, angioedemas and erythemas), 2) use of antihistamines, and 3) quality of life in 34 of these ASST+ patients as well as 21 ASST- CU patients. ASST+ and ASST- CU patients did not differ in disease severity assessing CU symptoms or quality of life (determined by Dermatological Life Quality Index, DLQI). However, ASST+ CU patients were found to exhibit substantially longer disease duration (9.1 vs. 2.5 years) and required significantly more antihistaminic medication (~3-fold) as compared to ASST- patients. Interestingly, anti-$Fc_\varepsilon RI$ and/or anti-IgE were found in very few CU patients (18% and 4%, respectively) and no significant differences were detected comparing ASST+ and ASST- CU patients for expression of either anti-$Fc_\varepsilon RI$ (ASST+: 18%, ASST-: 19%) or anti-IgE (ASST+: 6%, ASST-: 0%). These data support the notion that ASST+ CU patients suffer from a distinct subform of CU that is of longer duration and requires higher levels of antihistaminic medication than other forms of CU. As anti-$Fc_\varepsilon RI$ and/or anti-IgE were not found to be of major significance in the pathogenesis of CU in ASST+ patients, our findings encourage continued efforts to identify relevant circulating histamine releasing factors in the serum of ASST+ CU patients.

P021

Venous leg ulcers and apoptosis: A TIMP-3 mediated pathway ?

Y. Herouy[1], M. Idzko[2], T. Hildenbrand[1], M. Y. Ali[3], J. Clement[1], D. Ferrari[4], F. Di Virgilio[4], J. Norgauer[1]

[1]University Hospital, Department of Dermatology, 79104 Freiburg, Deutschland
[2]University Medical Center, Department of Pneumology, 79106 Freiburg, Deutschland
[3]University Medical Center, Department of Gastroenterology, 79106 Freiburg, Deutschland
[4]University of Ferrara, Department of Experimental and Diagnostic Medicine, Section of General Pathology, 44100 Ferrara, Italy

Proteolysis of cell surface- and extracellular matrix molecules (ECM) is intrinsically linked to cell function and fate. Tissue inhibitor of metalloproteinase-3 (TIMP-3) belongs to a family of secreted proteins that regulate the activity of several metalloproteinases and is an inducer of apoptotic cell death. The prodeath domain of TIMP-3 resides in its N-terminal region and apoptosis is induced by the activation of a signaling cascade involving caspase-8 and -9. Venous leg ulcers account for the majority of chronic wounds and lack clinical signs of necrotic events. Here we determined the protein expression of TIMP-3, FAS/CD95 by immunohistochemistry, the caspase-8 and caspase-9 protein expression and activity by caspase activity assays as well as the DNA-fragmentation by positive DNA in situ nick end-labeling (TUNEL) in tissue specimens of venous leg ulcers or healthy skin controls. Staining with anti-TIMP-3 displayed an intense signal around collagen bundles in the reticular dermis of venous leg ulcers. Staining with Fas/CD95 showed an intense cellular immunoreactivity in lesions in comparison with healthy controls. Immunoblotting with caspase-8 and -9 antibodies revealed prominent bands for the processed forms as well as the active subunits of these apoptosis regulators. Lesional skin displayed strongly increased activity for caspase-8 and caspase-9 in comparison with healthy controls measured by caspase activity assays. Moreover, quantification of apoptotic cells by TdT-mediated incorporation of dUTP into fragmented DNA (TUNEL) revealed significantly elevated apoptotic cell death in venous leg ulcers in comparison with healthy controls. In summary our data for the first time provide evidence that venous leg ulcers could be the result of an apoptotic signaling pathway, which might be induced through a TIMP-3-mediated inhibition of proteinase-dependent ECM-degradation. These results suggest that controlling programmed cell death may have therapeutic potential in preventing venous leg ulceration.

P022

Screening of Unselected Sebaceous Gland Neoplasias and Hyperplasias for Microsatellite Instability

R. Kruse[1], A. Rütten[2], N. Schweiger[1], E. Jakob[1], M. Mathiak[3], P. Propping[4], E. Mangold[4], M. Bisceglia[5], T. Ruzicka[1]

[1]Universität Düsseldorf, Hautklinik, 40225 Düsseldorf, Deutschland
[2]Labor für Dermatohistopathologie Friedrichshafen, 88048 Friedrichshafen, Deutschland
[3]Universität Bonn, Institut für Pathologie, 53127 Bonn, Deutschland
[4]Universität Bonn, Institut für Humangenetik, 53111 Bonn, Deutschland
[5]Casa Sollievo della Sofferenza Hospital San Giovanni Rotondo, Department of Pathology, 71013 San Giovanni Rotondo, Italy

Sebaceous gland neoplasias are the cutaneous manifestation of the Muir-Torre syndrome (MTS), which is known to be a phenotypical variant of hereditary non-polyposis colorectal cancer (HNPCC). Both HNPCC and MTS are caused by inherited DNA-mismatch-repair (MMR) defects. As a prominent molecular genetic feature all tumors associated with a DNA-MMR-defect exhibit high microsatellite instability (MSI-H). So far, the frequency of DNA-MMR-defects in patients selected solely on the basis of a sebaceous gland tumor has never been determined. In order to estimate this frequency, we assessed MSI with up to 10 microsatellite markers in a newly collected unselected series of 25 sebaceous gland neoplasias (6 sebaceous adenomas, 16 sebaceous epitheliomas, 3 sebaceous carcinomas) in comparison to 33 sebaceous gland hyperplasias from different unrelated patients. As many as 15 of the 25 sebaceous gland neoplasias (60%), but only one of the 33 sebaceous gland hyperplasias (3%), exhibited MSI-H. Thus, in our study, the majority of patients with a sebaceous gland neoplasia in contrast to patients with a sebaceous gland hyperplasia are highly suspicious for an inherited DNA-MMR-defect. On the basis of the subsequently collected tumor histories, 9 of the 15 patients with an MSI-H sebaceous gland neoplasia were identified to have Muir-Torre syndrome. However, no clinical diagnosis of HNPCC could be made in any of these patients. In the sebaceous tumors of the remaining 6 patients, MSI-H was an incidental finding. In comparison with MSI screening studies in a variety of other randomly selected tumors, our study identifies sebaceous gland neoplasias as tumors with the highest frequency of MSI-H reported so far, whereas the sebaceous gland hyperplasia rarely exhibits MSI-H. Therefore, screening for MSI in sebaceous gland neoplasias will be of high value for detection of an inherited DNA-MMR-defect which predisposes to various types of internal cancers.

P023

AP-1 Complex Composition in Oxysterol-treated Keratinocytes

M. Schmuth[1], K. Hanley[2], P. Lau[2], P. M. Elias[2], D. D. Bikle[3], K. R. Feingold[3]

[1]Universitaet Innsbruck, Dermatologie, A-6020 Innsbruck, Oesterreich
[2]UCSF, Dermatology, CA 94121 San Francisco, USA
[3]UCSF, Medicine, CA 94121 San Francisco, USA

Oxysterols stimulate keratinocyte differentiation, which involves the formation of the cornified envelope on the inner plasma membrane by transglutaminase crosslinking of several constituent proteins. We previously reported that oxysterols increase the expression of one of these crosslinked proteins, involucrin and that this effect can be abolished by mutations of the distal activator protein (AP)-1 response element in the involucrin promoter. Furthermore, oxysterols increase AP-1 binding in an electrophoretic gel mobility shift assay and induce the expression of an AP-1 reporter. In the present study, we further describe the individual components of the AP-1 complex, which are involved in the oxysterol-mediated AP-1 activation and stimulation of keratinocyte differentiation. We identified Fra-1 within the AP-1 DNA binding complex by super shift analysis of nuclear extracts from oxysterol-treated, cultured keratinocytes. Western blot analysis demonstrated that oxysterol treatment increased the levels of Fra-1 and Jun-D, while Northern analysis revealed that oxysterols increased mRNA levels for Fra-1, c-Fos and Jun-D. Fra-1 mRNA was also increased in vivo after topical treatment of hairless mice with oxysterols. Since oxysterols are known to activate liver X receptor (LXR), we searched for putative LXR-response elements (LXRE) in the 5' upstream regions of these genes and identified putative binding sites within the Fra-1 and Jun-D, but not the c-fos promoter regions. Together these results demonstrate that oxysterols stimulate involucrin expression by increasing the levels of specific proteins of the AP-1 complex, indicating that oxysterols regulate keratinocyte differentiation by inducing the AP-1 factors, which in turn activate the genes required for epidermal differentiation. The presence of LXREs within the promoter regions of individual AP-1 proteins suggests that these oxysterol effects may be mediated by LXR.

P024

The molecular basis of Fabry disease in Germany

T. Wiederholt[1,2], P. Poblete[1,2], F. Breunig[3], H. F. Merk[1], J. Frank[1,2]

[1]University Clinic of the RWTH, Dept. of Dermatology and Allergy, 52074 Aachen, Deutschland
[2]University Clinic of the RWTH, Interdisciplinary Center for Clinical Research (IZKF), 52074 Aachen, Deutschland
[3]University Clinic of Wuerzburg, Dept. of Internal Medicine, 97080 Wuerzburg, Deutschland

Fabry disease (angiokeratoma corporis diffusum) (OMIM 301500) is an X-linked recessive inborn error of glycosphingolipid metabolism, caused by deficiency of lysosomal alpha-galactosidase A (alpha-Gal A; EC 3.2.1.22). Due to the X-linked transmission of the disease most patients are hemizygous males. Reduction of alpha-Gal A activity results in the accumulation of neutral glycosphingolipids in a number of tissues including vascular endothelial cells. Genotype-phenotype correlations in this disorder have not as yet been fully elucidated. In the classical form of Fabry disease, mutations in the alpha-Gal A-gene result in an almost complete loss of catalytic activity of the encoded enzyme alpha-Gal A. Clinically these patients reveal diffuse angiokeratomas, acroparesthesia, and vascular disease of the kidneys. Heterozygous females are obligate carriers and clinically mostly unaffected. However, in some cases they might reveal symptoms as e.g. renal insufficiency or cardiomyopathy. Here, we studied the molecular basis of this rare condition in four unrelated German families for the first time. Using PCR and automated DNA sequencing we identified four different mutations in the alpha-Gal A-gene, consisting of one missense (P345S), one nonsense (W399X) and two frameshift mutations (717delAA, 1222delA). To date, several mutations in the alpha-Gal A-gene have been identified in other countries, the majority representing private mutations. Interestingly however, W399X and 717delAA have previously been reported in patients from Great Britain and the USA. Thus, these genetic alterations possibly reflect mutational hotspots or founder effects in this disorder. Our findings support the importance of molecular genetic studies in Fabry disease to identify affected family members, in particular because enzyme replacement therapy is available now.

P025

Thiopurine Methyl Transferase Antagonism - Phenotype versus Genotype in 1088 individuals

K. Önder[1], B. Paulweber[2], H. Hintner[1], J. Bauer[1]

[1]Landeskrankenhaus, Dermatologie, 5020 Salzburg, Oesterreich
[2]Landeskrankenhaus, 1. Medizin, 5020 Salzburg, Oesterreich

An excellent demonstration model for pharmacogenetics represents Thiopurine Methyl Transferase (TPMT) because the biotransformation property of thiopurine drugs by TPMT has been known for a long time. The relationship of toxicity and therapeutic efficacy of widely used thiopurine drugs like azathioprine depends on the TPMT genotype which has to be determined prior to the initiation of drug therapy. Earlier studies were either limited to genotype analyses of small amounts of patient groups or time and cost intensive enzyme activity tests. The results of these studies claimed 11 % heterozygosity and 0,3 % homozygosity of TPMT enzyme activity in various populations. Accuracy of statistic data can only be guaranteed if the analysed amount of information shows a significant dimension and therefore we performed extensive studies with 1088 unrelated persons by a compact genetic screen. We composed a fast PCR screen which allowed us to investigate different DNA-probes at one time and revealed crucial TPMT-mutation data. We detected 9,19 % heterozygote TPMT*3A mutations, 0,37 % heterozygote TPMT*3C mutations, but surprisingly no homozygote TPMT*3A or TPMT*3C mutation. In contrast to earlier published data which suggest 0,3 % homozygosity of enzyme activity of TPMT in a given population according to the Hardy-Weinberg theorem, this theoretical approach could not be reproduced on the genetic level.

P026

Genetic background of Chloroquine - treatment in Porphyria cutanea tarda

E. Köstler[1], U. Stölzel[2], A. Koch[1], M. Richter[3], C. Wittekind[4], A. Tannapfel[4], U. Wollina[1]

[1]Hospital Dresden-Friedrichstadt, Dresden, Department of Dermatology, 01067 Dresden, Deutschland
[2]Klinikum Chemnitz, Department of Medicine II, D-09116 Chemnitz
[3]Hospital Döbeln, Department of Medicine, 04720 Döbeln, Deutschland
[4]University of Leipzig, Institute of Pathology, 04103 Leipzig, Deutschland

The role of HFE gene mutations, which are associated with porphyria cutanea tarda (PCT), on the therapeutic response to chloroquine is unknown.
After recently defining HFE mutations (C282Y and H63D) in patients with PCT we retrospectively analyzed a data base of chloroquine-treated patients with PCT on whether these mutations might have influenced the clinical response, urinary porphyrin excretion, liver enzyme activities (ALT, AST) and serum iron markers. Sera and corresponding complete sets of data before and after therapy were available in 62 of 207 patients with PCT who were treated exclusively with chloroquine (125-250 mg twice weekly) between 1985 and 1999.
For treatment low dose chloroquine diphosphate, 125-250 mg twice weekly, was used during a median time of 16 months (range, 12-26). The majority (37/62=61%) of German PCT patients carry HFE mutations. Chloroquine therapy was accompanied by clinical remission and reduced urinary porphyrin excretion ($p < 0,001$) in the 24 (39%) patients with HFE wild type as well as in 34 (55%) HFE heterozygous patients with PCT. Decreases of serum iron markers following chloroquine therapy were limited to patients with PCT and HFE wild-type. All patients homozygous for the ^fi82Y mutation (3/62=5%) had high serum iron, ferritin and transferrin saturation and failed to respond to chloroquine treatment.
Conclusions: C282Y heterozygosity and compound heterozygosity of HFE mutations did not compromise the therapeutic response to chloroquine. Since HFE C282Y homozygotes (+/+) did not respond to chloroquine and decrease of serum iron markers was limited to patients with PCT and HFE wild-type, phlebotomy should be first line therapy in patients with PCT and HFE-mutations. These data suggest that a search for HFE-Mutation is necessary in porphyria cutanea tarda.

P027

Significant association of the -174 bp promotor polymorphism of the human interleukin 6 gene with severe type of alopecia areata

M. Stockmeier[1], N. Jetter[1], G. Messer[1]

[1]Ludwig-Maximilians University, Department of Dermatology and Allergology, 80337 München, Deutschland

Alopecia areata is a common disorder thought to be mediated by immunological mechanisms involving autoimmune paths. The exact pathophysiologic and genetic background still needs to be enruled. As a major factor in the specific immune response the pluripotent cytokine interleukin 6 (IL-6) provides strong proinflammatory impact on the development of an alopecia areata infiltrate. Recently, an allelic polymorphism (G to C transition) was described within the promoter region of the human IL-6 gene in position -174 bp neighboring a specific recognition motif for transcription factors. The CC haplotype was found associated to a stronger IL-6 response. Here we have analysed this mutation by restriction with the endonuclease *Nla*III after PCR amplification (5´-TTGTCAAGACATGCCAAGTGCT-3´ and 3´-primer: 5´-GCCTCAGAGAGACATCTCCAGTTCC-3´). 87 patients with alopecia areata, 44 of the multilocularis, 20 totalis and 23 universalis type were investigated and compared to 207 healthy individuals. All 87 were treated with diphencyprone (DCP). The totalis and universalis type was negatively associated to the frequent allele (GG) and showed significant association to the high response CC allele in alopecia areata universalis (80%; $p<0.05$, chi square test). So far, no association to the therapeutical response to DCP could be detected. The IL-6 promoter polymorphism might account for a stronger IL-6 cytokine response in patients with a more severe course of the disease. For analysis of the -174 bp-IL-6 polymorphism to the therapeutic response a larger collective of patients will be necessary.

P028

Efficient expression of the tuberous sclerosis 2 tumor suppressor gene by retroviral gene transfer

A. Vu[1], T. Licht[2], M. Gashghaeinia[1], R. Wienecke[1]

[1]Ludwig-Maximilians-University Munich, Department of Dermatology, 80337 München, Deutschland
[2]Technical University Munich, Department of Hematology and Oncology, 80333 München, Deutschland

Tumor-suppressor genes are frequently antiproliferative. Therefore cells expressing these genes do not grow well and it is difficult to express these genes over a sustained period of time. For example cells transfected with the tuberous sclerosis gene 2(TSC2) in the pcDNA3 construct express only very modest levels, of the TSC2 protein product tuberin.In order to achieve higher expression levels TSC2-was subcloned into the pLXIN vector. This bicistronic vector contains the G418 selection marker downstream of TSC2, so that G418 resistance is only achieved in cells expressing TSC2. In addition, retroviral gene transfer yielded a much higher transduction rate than the conventional gene tranfer by liposomal transfection. Defective retroviruses were generated through transient transfection of Phoenix-Eco 293T cells with retroviral pLXIN or pLXIN-TSC2 plasmids using the calcium phosphate transfection system (Gibco). For determining the infectious virus titer NIH3T3 cells (10^5 / 100 mm well) were once infected with 1 ml or the dilution of 10^{-1}-10^{-6} of supernatants of Phoenix-Eco 293T cells that were transfected with pLXIN or pLXIN-TSC2. Cells were selected in G418 for 2-3 weeks, then stained with methylene blue in 50% ethanol for counting the number of forming colonies. TSC2(-/-)REF were incubated with supernatants of Phoenix-Eco 293T cells that were transfected with pLXIN or pLXIN-TSC2 at a titer of 2.2 -10^3 colony forming units (CFU)/ml for 12 h on 2 consecutive days. After selection with 300 µg/ml G418 for 2 weeks, cells were tested for the expression of tuberin by immunoblotting.Immunoblotting demonstrated that high levels of tuberin were achieved. These cells also showed altered biological properties such as changes of cell shape and suppressed secretion of VEGF.

P029

Fumaric acid esters are potent immunosuppressants: inhibition of acute and chronic rejection in rat kidney transplantation models by methyl hydrogen fumarate

M. Lehmann[1], K. Risch[1], H. Nizze[2], J. Brock[1], H. Volk[3], K. Asadullah[4]

[1]University of Rostock, Institute of Medical Biochemistry and Molecular Biology, D-18057 Rostock,
[2]University of Rostock, Institute of Pathology, D-18057 Rostock
[3]Humboldt-University Berlin, University Hospital Charite, Institute of Medical Immunology, D-10117 Berlin,
[4]Schering AG, Research Business Area Dermatology, D-13342 Berlin

The effectiveness and safety of fumaric acid esters (FAEs) for the treatment of psoriasis has been demonstrated. Their mode of action however is poorly understood so far. To determine the immunomodulatory potential of Calcium methylhydrogenfumarate (CaMHF) in transplant models, we tested the compound in rat transplantation models of acute (AR) and chronic rejection (CR). Orthotopic kidney transplantation was combined with contralateral nephrectomy using the strain combination WF to BDIX (AR) and F344 to LEW (CR). Recipients were treated per os prophylactically (day -28 to +28, AR and CR model) or therapeutically (day +30 to +60, CR model). CaMHF significantly prolonged the onset of AR in the WF/BDIX model. The half life of the grafts in the CaMHF group was 14 d vs. 7 d in the placebo treated and 9 d in untreated controls ($p<0.01$). 3/10 CaMHF treated rats permanently survived (>100 d) resulting in mean survival time >42.3±41.0 ($p<0.01$) vs. >28.3±38.3 d (placebo-treated control) and 9.4±2.6 d (control). In the F344/LEW model of chronic graft injury, only prophylactic CaMHF treatment significantly inhibited the development of chronic graft dysfunction ($p=0.001$) (mean survival time >28.4±2.0 and >23.9±6.0 and 21.1±5.4 weeks in the prophylactic CaMHF, therapeutic CaMHF, and placebo group, respectively); by 30 weeks 6/10 prophylactically treated animals were still alive vs. 3/9 and 1/10 in the therapeutically treated and the placebo-treated group, respectively ($p<0.05$). These findings indicate that CaMHF-treatment is effective to inhibit AR and CR, demonstrating remarkably in vivo immunomodulatory efficacy. Thus, FAEs have to be considered as considerable immunosuppressive drugs. This might have implications with regard to safety issue (e.g. immune monitoring) and novel potentially suitable indications (e.g. transplantation and other immune diseases).

P030

T-cell stimulatory activity of *Propionibacterium acnes*

U. Jappe[1], J. Henwood[2], E. Ingham[2], K. T. Holland[2]

[1]Universität Heidelberg, Dermatologie, 69115 Heidelberg, Deutschland
[2]University of Leeds, Microbiology, LS2 9JT Leeds, United Kingdom

Previous investigations had revealed immunological properties of *Propionibacterium acnes*, the microorganism associated with inflammatory acne. The purpose of this study was to investigate whether *P. acnes* had only antigenic and mitogenic activity or additional superantigenicity. A lymphocyte transformation assay was used to detect responses to *P. acnes* whole cell isolates. Peripheral blood mononuclear cells from cord blood samples (CBMNC), which are believed to be immunologically naive, responded after incubation with stationary phase *P. acnes* cells. In order to determine the nature of T-cell reactions to *P. acnes* cells a mouse-anti-human MHC class II monoclonal antibody (TU39) was used in the lymphocyte transformation assay to inhibit antigenic stimulation of lymphocytes. The reaction of CBMNC to *P. acnes* cells was not suppressed completely by the blocking antibody. The analysis of the T-cell receptor (TCR) BV repertoire using flow cytometry of unstimulated and stimulated cells indicated that *P. acnes* induced no deletion or over-representation of certain BV element bearing T-cells. The TCRBV analysis was repeated after pre-incubation with the antigen stimulation blocking antibody. Deletion of T-cells bearing certain BV components occurred, and there was no over-representation of T-cells carrying certain BV components. Two mechanisms of lymphocyte activation by *P. acnes* cells were apparent, antigen and mitogen driven. TCRBV analysis was performed for the purified superantigen TSST-1 and *P. acnes* with interleukin-2 (IL-2), since it was possible that overstimulation of cells may have reduced their capacity to respond. There was overexpression for BV2 after incubation with TSST-1 together with IL-2 as expected for this superantigen, but similiar BV expressions were not detected after incubation with *P. acnes* and IL-2. The results suggested mitogenic but not superantigenic activity of *P. acnes*.

P031

Interactions between peptide-pulsed dendritic cells and tumor-specific T cells: Implications for dendritic cell-based immunotherapy

D. Dieckmann[1], B. Ring[1], L. Jenne[1], G. Schuler[1], E. Schultz[1]

[1]Dermatologische Universitätsklinik, 91052 Erlangen, Deutschland

Dendritic cell-based immunotherapy represents a promising approach to fight cancer as dendritic cells (DCs) play a key role in inducing anti-tumor immunity. Antigen loading of DCs can be easily performed with tumor-associated peptides. It has been shown that vaccination with peptide-pulsed DCs can induce tumor-specific CD8+ and CD4+ T cell responses even in patients with advanced cancer. To optimize the vaccination efficacy we studied the interactions between monocyte-derived, peptide-pulsed DCs and tumor-specific T cells in vitro. As expected, mature DCs stimulated tumor-specific CD8+ CTLs more efficiently than immature DCs. Surprisingly, and in contrast to data from murine studies, tumor-specific CD4+ T cells were stronger activated by immature DCs, possibly due to the presence of more empty or peptide-receptive HLA class II molecules on immature than on mature DCs. Furthermore, we could show that immature DCs express membrane-bound HLA-DM, a molecular chaperone involved in exogenous peptide-loading. Upon maturation DCs downregulate surface expression of HLA-DM. The observed difference in presentation of exogenous peptide between immature and mature DCs could be overcome by use of higher peptide doses. Peptide-pulsed DCs were recognized by antigen-specific CD4+ T cells up to 96h, indicating a prolonged stability of the HLA class II-peptide complexes. Moreover, peptide-pulsed DCs could be cryopreserved and thawed without loosing their T cell stimulating capacity. Finally, simultaneous loading of DCs with the antigenic peptide and an irrelevant peptide did not hamper the activation of tumor-specific T cells. These findings may have important implications for DC-based immunotherapy.

P032

Sequential expression of cytokines and receptors in the first 16h in L.major-infected footpads

J. Ehrchen[1,2], J. Roth[2], E. Nattkemper[2], T. Vogl[2], G. Varga[1], C. Sorg[2], C. Sunderkötter[1,2]

[1]UK Münster, Hautklinik, 48149 Münster, Deutschland
[2]UK Münster, Inst. für Exp. Dermatol., 48149 Münster, Deutschland

In exp. leishmaniasis the elaboration of Th1 cells in resistant C57Bl/6 and of Th2 cells in susceptible BALB/c mice depends on the cytokine milieu and on signals from APC. Strain-specific differences in cytokine expression are known for lymphnodes, but not for footpads, i.e. the site of infection, APC maturation and antigen uptake. By performing real time PCR, a method sensitive enough to detect gene expression by few cells, we analysed footpads for induction of factors with a potential to influence the immune response and for early strain-specific differences. We found that infection with *L.major* induced several cytokine genes, while two with possible relevance for the Th1/Th2 response (IL 18, TARC) were not altered. GM-CSF and TARC showed strain specific differences already prior to infection with higher expression in BALB/c mice. This is remarkable since both cytokines are associated with a Th2 response and as we could show that in BALB/c higher PMN numbers are associated with susceptibility. Within 8 hrs after infection there was a markedly higher induction of TNF-α, CCR1, and CXCR3 (and of osteopontin, as reported earlier) in C57Bl/6 than in BALB/c mice. CCR1 and CXCR3 are associated with recruitment of cells of the Th1 response, while TNF-α has been shown to be mandatory for induction of an efficient Th1 response against *L.major*. After 16hrs some of these differences already declined, while there emerged significantly stronger induction of MIP2 in C57Bl/6 and of IL5 in BALB/c. Remarkably, dermal expression of IL12 and IFN-γ, two important inducers of Th1 cells, did not regularly correlate with resistance. Starting to reveal the cellular sources of these mediators we found by in situ hybridization and quantification of locally released protein that the higher protein release of the Th1-inducing cytokine osteopontin in C57Bl/6 mice derived from keratinocytes. Thus, we could reveal for the first time that there are striking strain-specific differences in the expression of cytokines and chemokine receptors also at the site of infection, detectable already within 8 hrs. This reflects the local presence of a complex network of cytokines influencing the Th1/Th2 response which comes into effect early and involves not only leukocytes, but also keratinocytes.

P033

Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress Th2 cytokine production

I. Bellinghausen[1], B. Klostermann[1], I. Böttcher[1], J. Knop[1], J. Saloga[1]

[1]Univ.-Hautklinik Mainz, 55131 Mainz, Deutschland

Recently it has been established that CD4+CD25+ T cells with regulatory capacity are present in human peripheral blood inhibiting allogeneic proliferation and cytokine production of preactivated CD4+CD25- responder T cells. The aim of this study was to analyze in an allergen-specific setting whether such regulatory CD4+CD25+ T cells also exist and function normally in atopic individuals, especially concerning the inhibition of Th2 cytokines. For this purpose, CD4+CD25- or CD4+CD25+ T cells from grass or birch pollen allergic donors (mainly with rhinitis) were stimulated in the presence of autologous mature monocyte-derived allergen-pulsed dendritic cells and the preactivated CD4+CD25+ T cells were added to CD4+CD25- T cells during restimulation. CD4+CD25+ T cells from the majority of the patients investigated proliferated poorly, produced less cytokines and inhibited the proliferation as well as the Th1 (IFN-gamma) and Th2 (IL-4, IL-5) cytokine production of CD4+CD25- T cells, but not IL-10 production. The suppression of CD4+CD25- T cells by CD4+CD25+ T cells was at least partially antigen-unspecific and not reversible by anti-IL-10, anti-TGF-beta or anti-CTLA-4 mAb, but by IL-2. In some patients, preactivated CD4+CD25+ T cells reproducibly showed strong proliferative responses, produced higher amounts of IL-4 and IL-10 than CD4+CD25- T cells and suppressed only the IFN-gamma production of CD4+CD25- T cells. These data indicate that regulatory CD4+CD25+ T cells are present and functional in most atopic patients with allergic rhinitis and are able to inhibit Th1 as well as Th2 cells cytokine production.

P034

Receptors for *Leishmania*-uptake and their functional relevance: Comparison between macrophages and dendritic cells

F. Woelbing[1], C. Sunderkoetter[2], Y. Belkaid[3], A. Nigg[1], J. Knop[1], E. von Stebut[1]

[1]Johannes Gutenberg-Universität, Department of Dermatology, D-55131 Mainz
[2]University of Münster, Department of Dermatology, D-48149 Muenster
[3]National Institutes of Health, Laboratory of Parasitic Diseases, 20892 Bethesda, MD, USA

Previously, we have demonstrated that *L. major*-infected dendritic cells (DC) - unlike macrophages (MΦ) - release IL-12 and effectively vaccinate against progressive disease. Additionally, we observed that i) skin-DC take up only *L. major* amastigotes, whereas MΦ efficiently phagocytose amastigotes as well as promastigotes, ii) the number of ingested parasites per cell was much lower in DC compared to MΦ, and iii) the kinetics of *L. major* uptake were much slower in DC than in MΦ. These differences could partially be related to differences in the involved receptors. In this study, we initially confirmed that bone marrow-derived DC (BMDC) - like skin-DC - efficiently take up amastigotes (isolated freshly from infected BALB/c tissues), but not cultured promastigotes (30±10 vs. 2±1 % infected cells/18 hrs of coculture, $n \geq 4$, $p < 0.005$). We then analysed the receptors involved in amastigote uptake by BMDC. We have previously shown that uptake of parasites by CD18- (thus CR3-)deficient MΦ is dramatically reduced. In contrast, CR3-deficient BMDC generated from CD18-/- mice showed no alterations in *L. major* phagocytosis, independent of whether parasites were opsonized by complement or not. In addition, blocking mannan-binding receptors or DEC-205 was without effect. We next studied if opsonization by Ig is involved in *Leishmania* uptake. Amastigotes isolated from B-cell (μMT) or T- and B-cell deficient (SCID) mice devoid of surface Ig were not phagocytosed (3±2 and 4±2 %, $p < 0.02$ compared to amastigotes isolated from BALB/c mice). When, however, metacyclic promastigotes were preincubated with Ig-containing serum from infected mice, they were efficiently taken up by DC (11±4 %, $p < 0.05$ compared to complement-opsonized or untreated promastigotes). Our data suggest, that parasite uptake by DC is dependent on Fc-receptors and provide an explanation why *Leishmania*-infected DC - in contrast to MΦ - release IL-12. Our data may also explain why DC exhibit MHC class I and II-dependent presentation of *L. major*-antigens, whereas in MΦ phagocytosis of the parasite leads to class II-restricted antigen presentation.

P035

Vaccinia Virus as oncolytic virus: destruction via interference with the cytoskeleton but infection of bystander DC

S. Greiner[1], J. Humrich[1], P. Thumann[1], A. Steinkasserer[1], G. Schuler[1], L. Jenne[1]

[1]Universitätsklinik Erlangen-Nürnberg, Dermatologische Klinik, 91054 Erlangen, Deutschland

Some viruses, termed oncolytic, are capable to induce lysis or apoptosis of malignant cells. As Vaccinia viruses (VV) genetically modified to express melanoma associated antigens have been used to vaccinate melanoma patients in order to generate an anti-tumoral immunity, we wanted to determine in vitro the direct effect of VV on melanoma cells and the consequences for local Dendritic Cells (DC). Especially we wanted to probe, whether VV has a potential to serve as oncolytic virus and thus might be injected directly into the melanoma lesion to destruct the melanoma lesion and booster an anti-melanoma immunity at the same time. We found that the wild type VV strain Western reserve (WR) infected melanoma cells extremely efficiently and underwent a complete viral replication cycle. After 24 hours of infection melanoma cells lost adherance and the typical stretched phenotype of melanoma cells.

Confocal microscopy revealed the destruction of the cellular cytoskeleton. After three days of infection melanoma cells were apoptotic as shown by FACS analysis. When immature DC were added to these cultures they phagocytosed apoptotic melanoma cells but no maturation of the DC occurred as shown by FACS analysis and mixed leukocyte reactions. Furthermore, simultaneous infection of the DC with VV reduced their capacity to induce T-cell proliferation dramatically.

We conclude that although melanoma cells can efficiently be infected with vaccinia viruses most likely no anti-tumoral immunity will be initiated by the intratumoral injection of WR, as bystander DC are deactivated.

P036

***Leishmania*-uptake induces increased release of Th1 promoting cytokines by dendritic cells from resistant but not susceptible strains**

A. Nigg[1], F. Woelbing[1], S. Lopez Kostka[1], J. Knop[1], E. von Stebut[1]

[1]University of Mainz, Department of Dermatology, 55131 Mainz, Deutschland

Protective immunity against *Leishmania major* (*L. major*) in resistant (C57BL/6) mice is the result of Th1-development whereas susceptibility to *L. major*-infection (in BALB/c mice) is associated with disease mediating Th2-cells. We have previously reported that *L. major*-infected dendritic cells (DC) - unlike macrophages - release IL-12 and effectively vaccinate against progressive disease. We subsequently found that BALB/c DC produce significantly less IL-1α than C57BL/6 DC and treatment of BALB/c mice *in vivo* with IL-1α during T-cell priming induced an IL-12-dependent skewing towards Th1 immunity associated with reduced lesion sizes and decreased parasite burdens. These data suggested that DC are involved in the critical events leading to the determination of genetic disease outcome. We therefore performed a detailed analysis of strain-dependent differences in the production of Th1- (IL-12, IFNγ, IL-18, TNFα) and Th2- (IL-4, IL-10, TGFβ1) promoting cytokines by DC after infection with different life forms of *L. major in vitro*. Bone marrow-derived DC (BMDC) were generated in IL-4 and GM-CSF-supplemented media and harvested as immature DC on day 6. *L. major*-infected or otherwise stimulated BMDC from both resistant and susceptible mice released comparable amounts of IL-12p40, TNFα, TGFβ1 and IL-10, whereas production of IFNγ, IL-18 and IL-4 was not detectable by ELISA. We subsequently investigated the effect of the cytokine milieu on the cytokine secretion pattern of DC. Interestingly, continued treatment with IL-4 during coculture of DC with *L. major* led to an increase in IFNγ production by C57BL/6 but not BALB/c DC. In addition, decreased release of IL-12 was detected in the presence of IL-4 by DC from both strains, but to a much higher extent in BALB/c DC. In summary, our results confirm the important role that early IL-4 might play for the development of Th1-dependent protective immunity. We demonstrate the existence of strain-dependent differences in IL-4-responsiveness and cytokine release between DC generated from genetically *Leishmania*-resistant and -susceptible mice. Our results suggest that genetic determination of disease outcome in cutaneous leishmaniasis is controlled, at least in part, on the level of DC.

P037

Head to head comparison of different genetic and protein-based immunization strategies to induce tumor antigen-specific protective anti-tumor immune responses in vivo

S. N. Wagner[1], T. K. Weimann[2], M. Bifang[2], C. Wagner[1], P. Luehrs[1], A. Zemann[2], M. Goos[2], G. Stingl[1], A. Schneeberger[1]

[1]DIAID, Dept. of Dermatology, University of Vienna, A-1090 Vienna, Oesterreich
[2]Dept. of Dermatology, University of Essen, D-45122 Essen, Deutschland

MAAs represents an attractive target antigen for the development of antigen-specific immunotherapeutic strategies against malignant melanoma. The recent development of different methods of genetic vaccination with pDNA now raises the questions (i) what method of genetic vaccination with pDNA is best for the induction of protective anti-tumor immunity and (ii) whether this method is superior to conventional immunization strategies with protein antigen. We therefore analyzed in a head to head comparison different strategies of genetic immunization using pDNA encoding MAA Pmel17/gp100 (i.e. application by direct ic. injection and the gene gun) with a Baculovirus-generated recombinant full-length Pmel17/gp100 protein vaccine (given in CpG ODN or Freund?s adjuvant) for the induction of protective antigen-specific anti-tumor immunity in the experimental Cloudman M3/DBA/2 mouse melanoma model. All treatment strategies induced antigen-specific IgG responses, with induction of both the IgG1 and the IgG2a isotypes by protein vaccines and exclusively of the IgG1 isotype by genetic vaccines. Despite the induction of antigen-specific Th1-associated IgG2a isotype responses, mice immunized with protein vaccines did not show (i) any significant induction of antigen-specific T cell responses in CTL assays and (ii) any significant protection against a subsequent tumor challenge with Pmel17+ M3 melanoma cells. In contrast, mice genetically immunized by direct injection with mPmel17-encoding pDNA showed significant induction of antigen-specific T cell responses in CTL assays and antitumor protection ($p<0.001$ at day 15). This protection was completely abolished by in vivo CD4+/CD8+ T cell depletion. Genetic immunization by the gene gun approach did neither result in induction of antigen-specific T cell responses nor in significant antitumor protection. The results of this head to head comparison demonstrate that, for the induction of antigen-specific protective antitumor T cell responses in vivo, genetic immunization by direct injection of pDNA is superior to genetic vaccination by the gene gun approach as well as to immunization with recombinant autologous protein.

P038

Efomycine M leaves T-cell activation unaltered

W. H. Boehncke[1], T. Krahn[2], M. P. Schoen[3]

[1]Johann Wolfgang Goethe-Universitaet, Dermatologie, 60590 Frankfurt, Deutschland
[2]Bayer AG, 42096 Wuppertal, Deutschland
[3]Otto-von-Guericke Universitaet, Dermatologie, 39120 Magdeburg, Deutschland

Several established therapies as well as some biologicals under development for the treatment of psoriasis cause immunosuppression via interference with T-cell activation. This is particularly so for agents targeting adhesion molecules with co-stimulatory properties. We have recently described efomycine M, a specific small-molecule selectin inhibitor exhibiting substantial anti-inflammatory effects in several animal models for cutaneous inflammation. In a series of experiments, we adressed the effects of efomycine M on T-cell activation. Peripheral mononuclear cells were stimulated either with the bacterial superantigen toxic shock syndrome toxin (TSST) or with a cocktail of cytokines and mitogen (IL-2 and PHA). Subsequently, FACS analyses were performed for the expression of activation markers, and secretion of cytokines was measured utilizing a cytometric bead array (CBA) kit. Proliferation was measured by means of ^{3}H-TdR uptake. Three independent experiments were done in triplicate either in medium alone, or in the presence of cyclosporine A or efomycine M, respectively (10^{-6} to 10^{-9} M). As expected, cyclosporine A interfered with the expression of the skin-homing receptor CLA as well as several early and late activation markers, such as CD25, CD54, CD69, and HLA-DR. Moreover, cytokine secretion and proliferation of T-cells were also reduced. These effects were dose-dependent. In contrast, efomycine M did not exhibit any significant effects on these parameters up to a concentration of 10^{-7}M. At 10^{-6}M, however, a sudden and dramatic reduction of all parameters was observed. Trypan blue staining documented the toxicity of efomycine M at this concentration. In conclusion, efomycine M leaves T-cell activation by bacterial superantigens and cytokines/mitogenes unaltered. It might therefore turn out to be less immunosuppressive when applied *in-vivo* compared to other anti-psoriatic therapies either established or under development.

P039

Human CD4+CD25+ regulatory, contact-dependent T cells induce interleukin-10 producing, contact-independent type 1-like regulatory t cells

D. Dieckmann[1], C. H. Brütt[1], H. Ploettner[1], G. Schuler[1]

[1]Dermatologische Klinik mit Poliklinik des Universitätsklinikums Erlangen, 91052 Erlangen, Deutschland

It has been recently demonstrated that regulatory CD4+CD25+ CD45RO+ T cells are present in the peripheral blood of healthy adults and exert regulatory function similar to their rodent counterparts. It remains difficult to understand how the small fraction of these T cells, that regulate via direct cell-to-cell contact and not via secretion of immunosuppressive cytokines, could mediate strong immune suppression. Here we show, that human CD4+CD25+ T cells induce long lasting anergy and production of Interleukin-10 in CD4+ CD25- T cells. These anergized CD4+CD25- T cells then suppress proliferation of syngeneic CD4+ T cells via Interleukin-10 but independent of direct cell contact, similar to the so-called type 1 regulatory T cells (Tr1). This `catalytic` function of CD4+CD25+ T cells to induce Tr1-like cells helps to explain their central role for the maintenance of immune homeostasis.

P040

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

A. Schwarz[1], A. Maeda[1], K. Kernebeck[1], T. Schwarz[1]

[1]Universitaet Muenster, Hautklinik, 48149 Muenster, Deutschland

Epicutaneous application of haptens onto UV-exposed skin does not induce contact hypersensitivity (CHS) but causes hapten-specific tolerance. This tolerance is mediated via T suppressor cells (Ts), now renamed regulatory T cells, since i.v. injection of T cells from UV-tolerized mice into naive animals renders the recipients unresponsive to the respective hapten. In contrast when T cells from UV-tolerized mice are injected i.v. into syngeneic mice which had been already sensitized against the respective hapten, the CHS response is not suppressed. Thus it was concluded that 1. UV-induced Ts are only able to inhibit the induction but not the elicitation of CHS; 2. Ts are inferior to T effector cells and 3. active only in the absence of T effector cells. Sensitization takes place in the regional lymph nodes, while elicitation in the area of hapten application. We postulated that Ts injected i.v. may primarily locate to the lymph nodes and not to the periphery and therefore only suppress the induction but not the elicitation of CHS. Thus, we studied whether Ts inhibit the elicitation of CHS when injected into the area of the challenge. Splenocytes and lymph node cells were obtained from C3H/HeN mice which were tolerized against dinitrofluorobenzene (DNFB) by application of the hapten onto skin which was exposed to UV radiation. I.v. injection of cells ($1x10^{8}$) into naive syngeneic mice inhibited sensitization against DNFB in the recipients, but had no suppressive effect when injected i.v. into recipients which were already sensitized against DNFB. Next $5x10^{5}$ cells were injected into the ears of DNFB-sensitized mice. Injection of the cells itself did not cause significant ear swelling. 3 hours after injection challenge with DNFB was performed. Mice which received cells from naive donors exhibited pronounced ear swelling, while animals injected with cells from mice which were UV-tolerized against DNFB revealed a significantly reduced CHS response. Inhibition was hapten-specific since i.c. injection of DNFB-specific Ts into the ears of oxazolone-sensitized mice did not affect challenge with oxazolone. Together, these data demonstrate that UV-induced Ts can suppress the effector phase of CHS provided they are present in the area of challenge.

P041

Haptenated Antigens Are Critical for the Elicitation of an Adaptive Immune Response in a Murine Model of Leukocyte-Adhesion Deficiency 1

T. Peters[1,2], W. Bloch[3], S. Grabbe[4], C. Wickenhauser[5], D. Keß[1,2], R. Hinrichs[2], K. Addicks[3], T. Krieg[1], W. Müller[6], K. Scharffetter-Kochanek[2]

[1]Universität zu Köln, Klinik für Dermatologie und Venerologie, D-50924 Köln
[2]Universität Ulm, Klinik für Dermatologie Und Allergologie, D-89081 Ulm
[3]Universität zu Köln, Institut I für Anatomie, 50924 Köln, Deutschland
[4]Universität Münster, Klinik für Dermatologie, 48149 Münster, Deutschland
[5]Universität zu Köln, Institut für Pathologie, 50924 Köln, Deutschland
[6]GBF, Experimentelle Immunologie, 38124 Braunschweig, Deutschland

Leukocyte adhesion deficiency type 1 (LAD1) is a rare primary immunodeficiency syndrome caused by a lack of functional ß2 integrins. LAD1 patients suffer from recurrent bacterial infections, impaired wound healing and skin ulcers. Besides, only weak or absent vaccine responses have been observed.
We investigated the role of ß2 integrins in adaptive immune responses towards T-dependent antigens in ß2 integrin-deficient ($CD18^{-/-}$) mice, and show that absence of CD18 results in the disability to form basic secondary lymphoid structures such as germinal centers and immuno-logical synapses, as detected by immuno-histo-chemistry and ultrastructural ananlyses. Upon immunization with tetanus toxoid, $CD18^{-/-}$ mice generated only weak primary and secondary IgG responses, which is in accordance to reports on human and bovine LAD1. Despite distinct morphological alterations and the failure to respond to tetanus toxoid, we found that $CD18^{-/-}$ mice mounted robust hapten-specific primary and secondary immune responses when immunized with the highly haptenated antigen $(4\text{-hydroxy-3-nitrophenyl})_{21}$ acetyl chicken γ globulin. These were not dependent on high interleukin-6 (IL-6) serum concen-trations in $CD18^{-/-}$ mice, as could be demonstrated by means of a $CD18^{-/-}$ $IL\text{-}6^{-/-}$ mutant.
We hypothesize that the additional antigen-presenting capacity of B cells, which are recruited by haptenated antigens, is critical for the elicitation of adaptive immune responses in LAD1. These findings may argue in favour of a modification of vaccination strategies in immuno-deficient individuals.

P042

Expanded heat shock protein (HSP) receptor CD91 expressing antigen-presenting cells in psoriatic lesions show nuclear NF-κB, produce TNF-alpha, and are in close vicinity of HSP70

O. Boyman[1], H. P. Hefti[2], M. Suter[2], B. J. Nickoloff[3], F. O. Nestle[1]

[1]Universitätsspital Zürich, 8091 Zürich, Schweiz
[2]Veterinärvirologie Universität Zürich, 8057 Zürich, Schweiz
[3]Loyola University Medical Center, 60153-5385 Maywood, IL, USA

A recently developed mouse model of psoriasis, using xenotransplants of normal appearing skin from psoriasis patients onto Rag-knockout mice additionally deficient in type I and type II interferon receptors (AGR), allows the investigation of early events in the development of a psoriatic lesion. Here, we were interested in possible factors leading to the activation of antigen-presenting cells (APC) and subsequently to the formation of a psoriatic phenotype. Pattern recognition receptors are crucial for the activation of APC. Thus, we performed immunohistochemistry stainings in spontaneously converted psoriatic lesions with antibodies against Toll-like receptors (TLR) and HSP receptor CD91. Whereas TLR were not found to be up-regulated during psoriasis development, CD91 positive cells showed a marked increase which correlated well with the spontaneous formation of a psoriatic lesion ($r > 0.9$). By confocal microscopy immunofluorescence double stainings, CD91 expressing cells were shown to be mainly CD83 respectively CD1c positive APC. To check the activation status of these CD91 positive APC we performed immunofluorescence stainings for the transcription factor NF-κB. CD91 expressing APC showed nuclear NF-κB and were thus activated whereas adjacent keratinocytes failed to show nuclear NF-κB. In addition to being activated, these CD91 positive APC produced TNF-alpha, an important pro-inflammatory cytokine in the induction and maintenance of psoriasis, and were in close vicinity to HSP70, a potential ligand for CD91.
We conclude that CD91 expressing APC may contribute to early events of inflammation during development of psoriatic lesions.

P043

The Fast Chemokine Response of Lymphocytes is Accelerated in Patients with Atopic Dermatitis: Hyperreleasability of Preformed RANTES and Immediate De Novo Over-Production of MIP-1β

A. Ambach[1,2], J. Fang[3], A. Weren[1,2], B. Bonnekoh[1], W. König[3], B. Schraven[4,2], B. König[3], H. Gollnick[1]

[1]Otto-von-Guericke-University, Dep. Dermatology & Venereology, 39120 Magdeburg, Deutschland
[2]Otto-von-Guericke-University, Center for Immunological Research, 39120 Magdeburg, Deutschland
[3]Otto-von-Guericke-University, Institute of Medical Microbiology, 39120 Magdeburg, Deutschland
[4]Otto-von-Guericke-University, Institute of Immunology, D- 39120 Magdeburg,

Liberation of chemokines (CK) within minutes seems desirable in certain circumstances. We recently described two modes of the fast CK-response in lymphocytes: release of preformed RANTES and *de novo* synthesis of MIP-1β. Early data showed a RANTES-hyperreleasability in atopic dermatitis (AD). These studies were extended, the fast CK-response was compared in AD-patients and healthy controls (HC) .

Ficoll-isolated peripheral blood mononuclear cells of 9 AD-patients and 9 HC were stained with monoclonal antibodies against RANTES (2 clones), MIP-1β, CD8, CD4, and isotype controls. In addition, cells were stimulated with PMA and ionomycin, and CK-staining at 0, 10, 20, 30, 60, 90, 120, 180min was quantified in a FACScan. For ELISA, platelets were removed and CK-levels were determined in the supernatant.

At time point zero, 46±35% of AD-lymphocytes stained $RANTES^+$ (HC: 65±27%). Setting the percentage of $RANTES^+$ cells at 0 min to 100%, the spontaneous as well as the ionomycin/PMA-stimulated RANTES release were accelerated significantly in AD: *Without stimulation*, RANTES-staining was reduced down to 60% within 10 min in AD (HC: 98±19%, $p=0.03$). *With* ionomycin/PMA-stimulation, 50% of RANTES+ AD-lymphocytes released their CK in 10 min (HC: 60 min). In contrast, only 2% of lymphocytes stained $MIP\text{-}1\beta^+$ at time point zero. In AD-lymphocytes, after ionomycin/PMA-stimulation this percentage increased much faster as compared to HC. AD: 24±17%, 26±22%, and 49±31% after 10, 30 and 120 min, respectively. HC: 2±2%, 3±3%, 12±8% ($p<0.05$). The MIP-1β increase was blocked by puromycin. Changes over time of CK-levels in the supernatant confirmed these results.

Taken together, in AD-lymphocytes both types of the fast CK-response, i.e. release of preformed RANTES and *de novo* production of MIP-1β, seem to be accelerated.

P044

Upstream signaling events of ERK activation in dendritic cells by contact sensitizers

E. Valk[1], S. Zahn[1], J. Knop[1], D. Becker[1]

[1]Department of Dermatology, University of Mainz, D-55101 Mainz

The signaling mechanisms in antigen presenting cells (APC) in the sensitization phase of contact hypersensitivity are hardly known. The aim of this study was to elucidate early signaling events following stimulation with the contact sensitizer MCI/MI (5-chloro-2-methylisothiazolinone plus 2-methylisothiazolinone) in human dendritic cells as a model for epidermal APC. Activation of the MAP kinases ERK 1/2 and p38 in monocytes by contact sensitizers has been described before. In dendritic cells MCI/MI induced rapid and sustained phosphorylation of ERK 1/2 while activation of p38 was found to be delayed. It could be demonstrated that activation of ERK1/2 by MCI/MI depends on the Raf/MEK/ERK pathway by means of specific antibodies for phosphorylated MEK1/2 and c-Raf as well as inhibition of ERK phosphorylation by the MEK1/2 inhibitor PD98059. Surprisingly, Ras activation assay revealed no activation of Ras after stimulation with MCI/MI in comparison to stimulation with PMA indicating that MCI/MI induced ERK phosphorylation does not require activation of Ras. Furthermore the role of calcium was studied as alternative pathways leading to the activation of the ERK core cascade are described to be regulated by calcium. ERK phosphorylation was inhibited in the presence of the intracellular calcium chelator BAPTA-AM but not diminished by reduction of the extracellular calcium concentration using EGTA. Further studies with the calmodulin antagonists TFP and W13 suggest that calmodulin is not necessary for calcium dependent activation of ERK. Instead, inhibition of MCI/MI induced ERK phosphorylation by TMB-8 indicates that Inositol-P3-mediated calcium release from intracellular stores is involved in this pathway. The data obtained for MCI/MI could be confirmed using the strong contact sensitizer TNCB.
Our study provides evidence for the activation of the Raf/MEK/ERK pathway in DC by strong contact sensitizers and points to the role of Ras independent but calcium sensitive pathways probably involving elements like PLC and PKC.

P045

The UV-B radiation-induced inhibition of interferon-gamma caused keratinocyte activation is independent on endogenous IL-10 and other soluble mediators.

M. Friedrich[1], R. Holzmann[1], H. Piazena[1], W. Sterry[1], K. Wolk[2], R. Sabat[2,3], K. Asadullah[2]

[1]University Hospital Charité, Dept. of Dermatology and Allergy, 10117 Berlin, Deutschland
[2]Schering AG, RBA Dermatology, 13342 Berlin, Deutschland
[3]University Hospital Charite, Institute of Medical Immunology, 10117 Berlin, Deutschland

Ultraviolet (UV) irradiation represents a well established treatment modality for several inflammatory skin diseases. The aim of this study was to investigate the mechanisms of UV-B radiation (UVBR)-induced KC insentitivity towards IFN-γ. Flow cytometric analyses indicated that UVBR temporarily inhibits the IFN-γ-caused activation of primary keratinocytes (KC) and HaCaT cells as measured by reduced ICAM-1 (CD54) and HLA-DR upregulation. Western blot experiments suggested that this is mediated by inhibition of STAT-1 phosphorylation. Since release of interleukin (IL)-10 and other soluble molecules has been suggested to contribute to the immunosuppressive and anti-inflammatory effects of UVR further experiments were performed. Neither IL-10 neutralization nor IL-10 addition had any effect on the UVBR-induced inhibition of IFN-γ-caused ICAM-1 upregulation. Furthermore, the supernatant from UVB-irradiated cells failed to inhibit the IFN-γ-caused effects in non-radiated HaCaT cells. In addition, irradiated cells from whom the supernatant was withdrawn 4 hours after irradiation still showed a diminished IFN-γ-induced response after 24 hours. Thus, no soluble factors are involved in the UVBR-induced inhibition of IFN-γ-caused KC activation. Moreover, our further data suggests that specific intracellular factor mediates this action.

P046

Immunomodulating effect of iloprost on PBMC

P. Rehberger[1], P. Beckheinrich[1], E. Wandel[1], M. Sticherling[1]

[1]Universitätshautklinik Leipzig, Experimentelle Dermatologie, 04103 Leipzig, Deutschland

Iloprost represents a stable prostacyclin derivative and has been licensed for Raynaud phenomenon. It is extensively used for symptomatic treatment of systemic sclerosis. Besides effects on microvascular blood circulation, immunomodulating effects have been speculated upon.The aim of this study was to determine the immune modulating abilities of iloprost on peripheral blood mononuclear cells (PBMC). Human PBMC of 19 healthy donors were incubated with three different stimuli to cover different ways of T cell activation: non-specific (PHA), receptor mediated (IL-2) and antigen specific receptor mediated (tetanus toxoid). The concentrations of iloprost corresponded to plasma levels obtained after infusion of a therapeutic dosage. Activation of PBMC was evaluated by measuring the proliferation in a BrdU-ELISA and cytokine production by either an IFNγ or IL-4 ELISA.The effect of iloprost on the proliferation of PBMC showed great interindividual differences. From 19 donors tested 7 showed an increase of proliferation, 6 showed an inhibitory effect of iloprost and 6 were unchanged. Interestingly a correlation between age and modulation of iloprost was found. PBMC of younger donors (20-40 years) showed a stimulating effect whereas elder individuals (>50 years) reacted with inhibition of proliferation. These effects were concentration dependent.Iloprost had no influence on the cytokine production. Whereas IFNγ production in PHA stimulated cultures was always high but could not be modulated by iloprost; Il-4 was always below the detection limit of the ELISA.The data on cell proliferation indicate immunomodulating effects of iloprost which might add to the clinical effectiveness in systemic sclerosis and possibly other immunomodulated diseases.

P047

Differential interleukin-10 receptor expression in antigen-presenting cell populations: do dendritic cells escape from inhibitory mechanisms?

S. Haehling von Lanzenauer[1], K. Wolk[1], S. Kunz[1], B. H. Gruenberg[2], W. Doecke[1], U. Reineke[3], K. Asadullah[2], H. Volk[1], R. Sabat[1,2]

[1]Humboldt University Berlin, Institute of Medical Immunology, Medical School Charité, D-10098 Berlin, Deutschland
[2]Schering AG, Research Business Area Dermatology, D-13342 Berlin, Deutschland
[3]JERINI Bio Tools GmbH, D-12489 Berlin, Deutschland

Interleukin (IL)-10 is the most important immunosuppressive cytokine. Its receptor is composed of two different chains, the alpha and the beta-chain. We studied the IL-10 receptor alpha-chain (IL-10Rα) because of its central role in IL-10 binding and signaling. Blood monocytes, one major target of IL-10 *in vivo*, express high IL-10Rα levels compared to lymphocytic cells and various tissues. However, monocyte culturing in the presence of macrophage colony-stimulating factor (M-CSF), which leads to the further differentiation into macrophages (Mφ), provoked an increase of IL-10Rα expression. In contrast, the presence of IL-4 or granulocyte-macrophage colony-stimulating factor (GM-CSF), which combined favor type 1 dendritic cell (DC1) development, provoked a reduction of IL-10Rα expression. The different IL-10Rα expression levels on the monocyte-derived cell populations were reflected in their different responsiveness towards IL-10. Similar data were obtained with *in vivo* developed immature Mφ and DC1. Our studies suggest that, compared to monocytes and Mφ, DC1 activity is controled to a minor extent by IL-10. This not only contributes to a better understanding of the biology of DC1, that play a major role in the induction of primary immune responses, but also has implication on clinical vaccination strategies using antigen-presenting cells.

P048

Keratinocyte unresponsiveness towards interleukin-10: lack of specific binding due to deficient IL-10 receptor 1 expression

B. H. Gruenberg[1], M. Seifert[2], R. Sabat[2,1], P. Donner[3], G. Gruetz[2], H. Volk[2], K. Wolk[1], K. Asadullah[1]

[1]Schering AG, Research Business Area Dermatology, D-13342 Berlin, Deutschland
[2]Humboldt University Berlin, Institute of Medical Immunology, Medical School Charité, D-10098 Berlin, Deutschland
[3]Schering AG, Proteinchemistry, Enabling Technologies, D-13342 Berlin, Deutschland

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

P049

Relevance of the low-affinity type of the Fcγ-Receptor IIIa-polymorphism in bullous pemphigoid

P. Weisenseel[1], S. Martin[1], K. Partscht[1], G. Messer[1], J. C. Prinz[1]

[1]Ludwig-Maximilians-Universität, Klinik und Poliklinik für Dermatologie und Allergologie, 80337 München, Deutschland

Bullous pemphigoid (BP) is mediated by autoantibodies directed against molecules of the basement membrane zone. The biological function of antibodies involves binding to Fc-receptors expressed on human leucocytes. Recent studies suggested that a functional single-nucleotide-polymorphisms (SNP) of the Fcγ-receptor IIIa (FcγRIIIa) at nucleotide 559 might predispose to the development of antibody-mediated autoimmune disorders such as SLE. This allelic difference affects the level of receptor affinity by predicting either a phenylalanine (F 158, low) or valine (V 158, high). We investigated if inherited frequencies of the high- and low-affinity FcγRIIIa polymorphism differed between patients with BP and healthy subjects.
Genomic DNA was extracted from peripheral white blood cells according to standard methods. FcγRIIIa polymorphism at nucleotide 559 was determined by an established polymerase chain reaction using different primers for the 158 V and F allele amplifying a specific 160 bp fragment, followed by agarose gel electrophoresis, ethidium bromide staining, UV-illumination and photographic documentation. Two primers from the T-cell receptor Vα22 gene amplified a fragment of 270 bp and were used as an internal positive control.
67 Caucasian patients with BP and 83 healthy controls were included into the study.
14,9% of the BP-patients were homozygous for the high-affinity FcγRIIIa allele(158 V). 58,2% of the BP patients and 45,8 % of healthy individuals were homozygous for the low-affinity (F)-type allele (odds ratio 1,65). The frequencies of the polymorphism in the control group were in range of formerly published frequencies for healthy Caucasian subjects.
According to the Odds ratio the low-affinity type of the FcγRIIIa-polymorphism (F) shows an association with BP when compared to the control group, that, however, was not yet statistically significant. Association of the 158 polymorphism with the clinical course of BP will have to demonstrate whether it might contribute to severity of the disease in a larger molecular epidemiology effort.

P050

Immune Cells as Sources and Targets of the Interleukin-10 Family Members?

K. Wolk[1], S. Kunz[1], K. Asadullah[1], R. Sabat[2,1]

[1]Schering AG, Research Business Area Dermatology, D-13342 Berlin, Deutschland
[2]Humboldt University Berlin, Institute of Medical Immunology, Medical School Charité, D-10098 Berlin, Deutschland

Our study investigated the expression of five novel human molecules with homology to Interleukin (IL)-10 and their receptors in blood mononuclear cells. IL-19 and IL-20 were found to be preferentially expressed in monocytes. IL-22 and IL-26 (AK155) expression was solely detected in T cells, especially upon type 1 polarization, and in NK cells. IL-24 (melanoma differentiation-associated gene 7) expression was limited to monocytes and T cells. Detection of these molecules in lymphocytes was predominantly linked to cellular activation. Regarding T cells, IL-26 was primarily produced by memory cells, and its expression was independent on costimulation. In contrast to the high expression of receptors for IL-10 homologs in different tissues and cell lines, monocytes and NK, B, and T cells showed clear expression only of IL-10R1, IL-10R2, and IL-20R2. In these cells, IL-20R2 might be part of a still-unknown receptor complex. As a result, immune cells may represent a major source but a minor target of the novel IL-10 family members.

P051

Identification of immunodominant T cell epitopes of the extracellular domain of desmoglein 3 in patients with pemphigus vulgaris and healthy individuals.

C. M. Veldman[1], K. Gebhard[1], A. Mainka[1], G. Schuler[1], M. Hertl[1]

[1]Dermatologische Universitätsklinik Erlangen, 91054 Erlangen, Deutschland

Pemphigus vulgaris (PV) is mediated by autoantibodies (Ab) against the desmosomal glycoprotein desmoglein 3 (Dsg3). Previous studies from our group and others showed that autoreactive Th1 and Th2 cells against Dsg3 are detectable in pemphigus patients supporting the idea that autoreactive T cells are critical for the induction and regulation of Ab production. Noteworthy, healthy carriers of the PV-prevalent HLA class II alleles, HLA-DRß1*0402 and HLA-DQß1*0503, showed an exclusive Th1 cell response against Dsg3 which was also restricted by these PV-associated HLA class II alleles. The aim of this study was to characterize the fine specifity of T cell recognition of Dsg3 in PV patients and healthy controls. A total of 24 Dsg3-reactive Th1 and Th2 clones (isolated by MACS secretion assay) from six PV patients and five healthy donors were co-cultured with 17mer peptides of the extracellular domains (EC) 1-5 of Dsg3 and APC expressing the HLA class II alleles HLA-DRß1*0402 or HLA-DQß1*0503 and the proliferative T cell response was determined by the incorporation of 3H-thymidine. Both, Th1 and Th2 cells from the PV patients recognized a limited set of epitopes that were located in the EC1 (DG2), EC2 (DG3, DG4, DG5) and EC3 (DG6, DG7) of Dsg3. Dsg3-reactive T cells from the healthy donors were mostly responsive to Dsg3 peptides DG2, DG5 and DG8 located in the EC1, EC2 and EC5 of Dsg3, respectively. All the identified Dsg3 peptides had similar or identical residues at positions 1 and 4 and some of them also at position 6 which presumably represent anchor motifs to the restricting HLA class II alleles. These findings strongly suggest that T cell recognition of Dsg3 is restricted to limited epitopes of the EC of Dsg3 and that differential epitope recognition of Dsg3 in patients and healthy donors may contribute to the development of autoimmunity. The identified immunodominant T cell epitopes of Dsg3 will be utilized for further therapeutic immune interventions aimed at restoring tolerance against Dsg3.

P052

Transiently reduced availability of peripheral blood lymphocytes (TRAP) induced by r848 potently inhibits hapten induced ear swelling in mice

M. Gunzer[1,2], H. Riemann[2], Y. Basoglu[2], S. Grabbe[2]

[1]Gesellschaft fuer Biotechnologische Forschung mbH, NG Immundynamik, 38124 Braunschweig, Deutschland
[2]Universitaet Muenster, Hautklinik/Zellbiologie, 48149 Muenster, Deutschland

R-848 is a more potent derivative of Imiquimod, the active substance of ALDARA™, exhibiting profound influence on B cells and dendritic cells in vitro and on the production of inflammatory cytokines in vivo. Since topical R848 has proven to be effective against localized skin tumors, we intended to test the effect of systemic R848 as a possible route of administration for metastasized tumors. Balb/c mice were sensitized with DNFB or FITC on the shaved abdomen. To investigate the effects of R848 on the sensitization phase of contact-hypersensitivity (CHS) responses, the drug was given three times i.p. during this sensitization phase. 5 days (FITC 6 days) later a hapten specific ear swelling response was elicited by painting DNFB/FITC on one ear. Where applicable, R-848 was given three times i.p. during this elicitation phase. 24 and 48 h later the relative ear swelling was measured. Samples of ears were taken for histologic examination. Animals were also serially bled from the tail vain for the analysis of peripheral blood. R-848 strongly increased the CHS sensitization to DNFB and FITC, even at low doses of hapten, which normally did not induce measurable responses. Unexpectedly, R-848 strongly decreased immune responses, even to the highest doses of hapten, when present during the CHS elicitation phase. Ears showed a clearly reduced cellular infiltrate in suppressed hosts, especially lacking CD4 and CD8 cells. Suppression of CHS reactivity was tightly associated with drug administration, as 24 h later normal ear swelling could be induced. Analysis of peripheral blood lymphocyte counts showed, that as early as 1 h after R848 administration >90% of CD4 and CD8 cells were depleted from peripheral blood. T cell counts recovered to normal levels within 48 h (TRAP), explaining the dependence of TRAP on the presence of active R848 in the animals. Thus, depending on the time and route of administration R848 can be a potent immune activator or supressor. The previously not recognized TRAP effect might represent an immune controlling function with potentially little side effects, since neither T cell viability nor generation of effector cells was affected by TRAP.

P053

Detection of identical T cells in multiple metastases of melanoma patients

D. Schrama[1], E. Fuchs[1], E. Broecker[1], J. C. Becker[1]

[1]Universitaet Wuerzburg, Hautklinik, 97080 Wuerzburg, Deutschland

For an efficient cellular immune response to metastatic tumors it is necessary that specific T cells circulate throughout the body. Surprisingly, in murine tumor models such a circulation could only be observed after treatment with IL2. In accordance to these findings in the mouse, a recent report demonstrated predominantly localized immune responses in untreated melanoma patients. Thus, we characterized in humans the effect of immune therapy on T cell infiltrates with a focus on the distribution of clonotypic T cells by clonotype mapping. This method is based on RT-PCR and denaturing gradient gel electrophoresis allowing to identify T cell clonotypes by virtue of their identical sequence. Advantages of this method is the ability to analyze the total repertoire and the detection threshold of 0.1% clonal expansion. Furthermore, based on specific primers for the β variable families and β constant region of the TCR, a real time PCR was established allowing semiquantitative analysis of the T cell repertoire. To this end, we analyzed 25 metastases of 4 melanoma patients receiving different immunotherapies. Each metastasis harbored an oligoclonal T cell infiltrate with varying clone numbers within a patient and overall between 2 and 33. The average number of clones/metastasis was 7.4 to 20.3 with a total average of 12.7. Furthermore, identical T cell clonotypes were present in several metastases of the same patient belonging mostly to moderate- or over-expressed BV families, the extend ranged from 5.4 to 21% of the total T cell clonotypes. Quantitative analysis of selected cases revealed a trend towards the usage of different BV families within a patient. As identical T cell clonotypes were observed in a couple of metastases excised after immune therapy during chemotherapy, we extended our analysis to an additional 6 metastases of two patients receiving solely chemotherapy. As expected, the metastases were characterized by an oligoclonal T cell infiltrate with clone numbers of 9-32. The average clone number/metastasis was 16.5. Surprisingly, however, identical T cell clonotypes were also observed in these metastases and the extend was similar to the observed ones in the patients receiving immunomotherapies, namely 8.7 and 15.1%. Therefore, chemotherapy seems to possess immunomodulating activity, i.e., it has at least the same impact on the distribution of T cells than immunotherapy.

P054

Long-term IL-10 presence induces the generation of a new APC population

C. Schoenbein[1], W. Doecke[1], K. Wolk[1], G. Gruetz[2], M. Jung[2], H. Volk[2], K. Asadullah[1], R. Sabat[1]

[1]Schering AG, RBA Dermatology, 13342 Berlin, Deutschland
[2]Humboldt University Berlin, Institute of Medical Immunology, 10098 Berlin, Deutschland

Interleukin (IL)-10 is one of the most crucial regulators of the immune system. However, little is known about the effects of long-term IL-10 presence *in vivo* and *in vitro*. This is of considerable importance, since high IL-10 levels are present for a long period in some diseases such as persistent viral infections, certain cancers and chronic stress. Our study investigated the influence of long-term IL-10 presence on monocytes *in vitro*. Already the treatment for one week induced the development of a new cell population characterized by its specific morphology and function: These cells showed high HLA-DR and strongly decreased CD86 cell surface expression compared to untreated controls. Surprisingly, mRNA levels of both HLA-DR and CD86 were increased. In contrast to macrophages and dendritic cells these cells produced high levels of IL-10, and low levels of IL-12 and TNF-α. Furthermore, these cells showed strongly diminished T-cell stimulation capacity, and even induced anergy in CD4+ T cells. Moreover, long-term IL-10-treated monocytic cells provoked phenotypic alterations in freshly isolated monocytes dependent on cell-cell contact. These results not only complement ours knowledge about immunosuppressive effects of IL-10 but also contribute to a better understanding of the mechanisms of persistent immunodeficiency.

P055

Expression of β7-integrin and GITR defined distinct subsets of human CD25$^+$ regulatory T cells

S. Fondel[1], E. Schmitt[2], J. Knop[1], A. H. Enk[1], H. Jonuleit[1]

[1]University of Mainz, Department of Dermatology, 55101 Mainz, Deutschland
[2]University of Mainz, Institute of Immunology, 55101 Mainz, Deutschland

CD25$^+$ regulatory T cells play an important role for the maintenance of antigen-specific tolerance and are critical for prevention of autoimmunity. However, populations of human CD4$^+$CD25$^+$ T cells isolated from the peripheral blood are heterogeneous and include T cells with regulatory properties as well as conventional CD4$^+$ T helper cells. Recently, gene expression analysis has revealed that $\alpha_E\beta$7-integrin and GITR (glucocorticoid induced TNF receptor family related antigen) are differentially expressed on murine regulatory T cells. In this study, we investigated whether human CD25$^+$ regulatory T cells can be identified by the expression of β7-integrins and/or GITR. We demonstrate here that the expression of β7-integrin and GITR defined two distinct subset of CD25$^+$ regulatory T cells with strong suppressive activities. Both subsets, β7-integrin-positive and GITR$^+$ CD25$^+$ T cells sorted from freshly isolated CD4$^+$CD25$^+$ T cells showed a constitutive expression of intracellular CTLA-4 and inhibited proliferation and cytokine synthesis of conventional CD4$^+$ T cells in a cell contact and activation dependent manner. In contrast, after depletion of GITR$^+$ and β7-integrin-positive cells, the depleted CD25$^+$ T cell population showed a normal proliferative capacity and cytokine production and no suppressive activity *in vitro*. Collectively, this results indicates that distinct subsets of human peripheral CD25$^+$ regulatory T cells can be identified by their surface expression GITR and β7-integrin.

P056

Mass Mapping and Tandem-Mass Spectrometry Reveals Polymorphism and a Novel Variant of Psoriasin

J. Bartels[1], J. Schröder[1]

[1]Universitätsklinikum Kiel, Klinische Forschergruppe der Hautklinik, 24105 Kiel, Deutschland

We recently purified from healthy human heel stratum corneum an *E. coli* killing 11 kD protein which could not be sequenced by Edman degradation. Peptide mapping and subsequent Edman degradation unambiguously revealed identity with the S100 protein Psoriasin.

Careful re-examination of the purification protocol revealed at least 6 psoriasin peaks upon cation exchange HPLC, which by reversed phase HPLC could be further divided into three additional separate peaks indicating at least 18 separable psoriasin variants. Electrospray ionisation (ESI) mass spectrometry (MS) revealed additional heterogeneity revealing masses from 10355 to 11486 Da. The most prominent psoriasin forms displayed masses of 11365, 11398 and 11216 Da. Mass mapping and Tandem-ESI-MS of trypsin-digested reduced and alkylated psoriasin forms revealed that the most abundant 11365 Da variant is identical with the NH_2-terminally acetylated common form of psoriasin that is missing the NH_2-terminal methionine. The 11216 Da mass represents a novel psoriasin variant differing in six amino acids compared to the common form, again NH_2-terminally acetylated without the methionine.

Future investigations will determine the biological significance of the psoriasin heterogeneity in particular with respect to the *E.coli*-selective antimicrobial activity.

P057

Psoriasin expression in normal and diseased skin

E. Wandel[1], B. Rosin[1], J. Schröder[2], R. Gläser[2], M. Sticherling[1]

[1]University of Leipzig, Department of Clinical and Experimental Dermatology, 04103 Leipzig, Deutschland
[2]University of Kiel, Clinic of Dermatology, Venerology and Allergology, 24105 Kiel, Deutschland

Psoriasin was initially identified as a highly abundant protein belonging to the S100gene family, expressed by abnormally proliferating keratinocytes in psoriatic epidermis. Subsequently, it was also found to be up-regulated in abnormally differentiating keratinocytes according to different tumour types. Psoriasin is a secreted protein that may exert an effect as a chemotactic factor for inflammatory cells. Very recently psoriasin has been shown to display antimicrobial activity against E. coli by depletion of zinc ions.
In the present study the expression of psoriasin was determined by in situ hybridisation using a specific probe generated in the lab and immunohistochemistry using a commercial monoclonal antibody in normal human skin of different localisation and in several inflammatory skin disorders. Expression of psoriasin mRNA was constitutively high in epidermis of normal skin whereas immunoreactivity was found predominantly in areas which are highly exposed to both, microbial challenge and UV-radiation (e.g. nose, forehead).
In inflammatory skin diseases the mRNA expression of psoriasin in the epidermis was generally high and increased immunoreactivity was found in psoriasis, follikulitis and condyloma acuminata whereas patients with lichen ruber or eczema showed only little staining in well defined parts of the epidermis.
This study demonstrates the modulation of psoriasin expression in both infectious and non-infectious skin diseases. The distinct expression in psoriatic skin explains the clinical experience of a low incidence of bacterial infection as well as indicating biological functions of the protein yet unknown, but beyond antibacterial activity.

P058

A local inflammatory response is required for efficient CD8+ T cell-mediated destruction of melanocytes in the skin following genetic immunization against the melanosomal protein TRP2

S. Montag[1], J. Brück[1], J. Steitz[1], J. Lenz[1], G. Reinhard[1], S. Büchs[1], T. Tüting[1]

[1]Klinik für Dermatologie der Universität Bonn, 53105 Bonn, Deutschland

Previously, we reported that gene gun-immunization of C57BL/6 mice with plasmid DNA encoding the xenogeneic, human melanosomal enzyme tyrosinase-related protein 2 (TRP2) resulted in the stimulation of antigen-specific cellular and humoral immunity cross-reactive with murine TRP2. ELISPOT assays revealed the induction of CD8+ T-cells in vivo recognizing the H2-Kb-binding peptide SVYDFFVWL which derives from an evolutionary conserved region of both murine and human TRP2 corresponding to amino acids 180-188 (TRP2aa180-188). Interestingly, fur depigmentation as a result of autoimmune-mediated destruction of melanocytes was always observed in the shaved and bombarded skin on the abdomen and only occasionally spread to other areas after significant delay. By combining gene gun bombardment of the skin with injections of recombinant adenovirus encoding human TRP2 we could show that the gene gun-induced inflammatory response significantly supports melanocyte destruction. Furthermore, genetic immunization with cDNA encoding a fusion protein between enhanced green fluorescent protein EGFP and the peptide TRP2aa180-188 strongly suggested that TRP2aa180-188-specific CD8+ T cells mediate melanocyte destruction. Currently, we are analyzing the mechanisms involved by immunohistopathology using different inflammatory stimuli to attract T cells into the skin. These results are also of considerable importance for the development of melanoma vaccines because they indicate that local proinflammatory signals in the tumor tissue need to be provided in addition to systemic stimulation of melanoma specific cytolytic T cells.

P059

New evidence for substance P as a modulatory neuropeptide in autoimmune hair loss

F. Siebenhaar[1,2], A. A. Sharov[1], T. Y. Sharova[1], M. Maurer[2], V. A. Botchkarev[1]

[1]Boston University School of Medicine, Dermatology, 02118 Boston, MA, USA
[2]University Hospital Mainz, Dermatology, 55131 Mainz, Deutschland

Increasing evidence suggests that the immune and nervous systems are closely integrated to modulate defense mechanisms within the cutaneous environment. Skin and particularly hair follicles are highly innervated by neuropeptide containing nerve fibers. Since substance P (SP) was shown to have the ability to modulate immune cell function via its NK-1 receptor and to modulate hair growth in healthy skin, we investigated the effect of SP in the C3H/HeJ mouse model for autoimmune hair loss (alopecia areata, AA). We found that SP is expressed in dermal nerve fibers, and that NK-1 receptor is expressed in hair follicle keratinocytes, peri- and intrafollicular macrophages, and CD4+ cells. After SP treatment we observed a remarkable decrease in the cellular infiltrates around affected hair follicles. As assessed by immunohistochemistry, there were significant decreases in the numbers of intrafollicular CD4+ ($p<0.05$) and CD8+ ($p<0.01$) lymphocytes as well as MOMA-2 positive macrophages ($p<0.01$), and a higher rate of activated mast cells was found after SP administration in AA affected mice, compared to vehicle treated controls. Furthermore, we detected a higher co-expression of the NK-1 receptor and MOMA-2 positive cells in AA affected skin treated with SP, indicating that SP might mediate its function via increased NK-1 receptor expression on macrophages in AA affected skin. These data provide new evidence for substance P as a modulatory neuropeptide within the skin immune system and raise a possibility for using substance P in alopecia areata treatment.

P060

Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T cell stimulatory capacity of human dendritic cells.

E. Panther[1], S. Corinti[2], M. Idzko[1], Y. Herouy[1], M. Napp[1], G. Girolomoni[2], J. Norgauer[1]

[1]University of Freiburg, Department of Experimental Dermatology, 79104 Freiburg, Deutschland
[2]Istituto Dermopatico dell`Immacolata, 00167 Roma, Italien

Dendritic cells (DC) express functional purinergic type 1 receptors, but the effects of adenosine in these antigen presenting cells have been only marginally investigated. Here, we further characterized the biological activity of adenosine in immature DC (iDC) and lipopolysaccharide (LPS)-matured DC (mDC). Chronic stimulation with adenosine enhanced the macropinocytotic activity and the membrane expression of CD80, CD86, MHC class I and HLA-DR molecules on iDC. Adenosine also increased LPS-induced CD54, CD80, MHC class I and HLA-DR molecule expression in mDC. In addition, adenosine dose-dependently inhibited tumor necrosis factor α and interleukin (IL)-12 release, whereas enhanced the secretion of IL-10 from mDC. The use of selective receptor agonists revealed that the modulation of the cytokine and cell surface marker profile was due to activation of A_2 adenosine receptor. Functionally, adenosine reduced the allostimmulatory capacity of iDC, but not of mDC. More important, DC matured in the presence of adenosine had a reduced capacity to induce Th1 polarization of naive $CD4^+$ T lymphocytes. Finally, adenosine augmented the release of the chemokine CCL17 and inhibited CXCL10 production by mDC. In aggregate, the results provide initial evidence that adenosine diminishes the capacity of DC to initiate and amplify Th1 immune responses.

P061

The influence of lysophosphatidic acid on the functions of human dendritic cells.

E. Panther[1], M. Idzko[1], S. Corinti[2], Y. Herouy[1], G. Girolomoni[2], J. Norgauer[1]

[1]University of Freiburg, Department of Experimental Dermatology, 79104 Freiburg, Deutschland
[2]Istituto Dermopatico dell`Immacolata, 00167 Roma, Italien

Lysophosphatidic acid (LPA) is a bioactive lipid mediator, which is generated by secretory phospholipase A_2. Here we studied the biological activity of LPA on human dendritic cells (DC), which are specialized antigen presenting cells characterized by their ability to migrate into target sites and secondary lymphoid organs in order to process antigens and activate naive T cells. We show that immature and mature DC express the mRNA for different LPA receptors such as EDG-2, EDG-4 and EDG-7. In immature DC, LPA stimulated pertussis toxin-sensitive Ca^{2+} increase, actin-polymerization and chemotaxis. During the maturation process, DC lost their ability to respond towards LPA with Ca^{2+} transients, actin polymerization and chemotaxis. However, LPA inhibited in a pertussis toxin-insensitive manner the secretion of interleukin 12 and tumor necrosis factor a as well as enhanced secretion of IL-10 from mature DC. Moreover, LPA did not affect the endocytic or phagocytic capacities and the surface phenotype of DC, while it increased the allostimulatory function of mature DC and inhibited their capacity to induce Th1 differentiation. In summary, our study implicates that LPA might regulate the trafficking, cytokine production and T cell activating functions of DC.

P062

Agonists of Proteinase-activated Receptor-2 (PAR-2) have Effects on Human Neutrophil Degranulation, Expression of Cell Adhesion Molecules and Transendothelial Migration

V. Shpacovitch[1], G. Varga[1], A. Strey[2], A. Lechtermann[3], J. Buddenkotte[1], S. Menzel[1], C. Sommerhoff[4], F. Mooren[3], V. Gerke[2], T. Luger[1], M. Steinhoff[1]

[1]University of Muenster, Dep.of Dermatology, 48149 Muenster, Deutschland
[2]University of Muenster, ZMBE, Dep.of Biochemistry, 48149 Muenster, Deutschland
[3]Institute of Sport Medicine, 48149 Muenster, Deutschland
[4]University of Munich, Clinical Chemistry and Clinical Biochemistry, 80336 Munich, Deutschland

Human neutrophils play a key role as a part of the immune response to microbal infections and during many inflammatory skin diseases. One of their main functions is the rapid killing of bacteria or fungi before they multiply and spread throughout the body. The main physiological and pathogenic activities of neutrophils are: 1) adherence and migration; 2) degranulation and release of inflammatory mediators; 3) phagocytosis and apoptosis. The expression of proteinase-activated receptor-2 (PAR-2) by human neutrophils has been demonstrated in previous studies, but the role of PAR-2 in biological functions of human neutrophils remains unclear. PAR-2 belongs to a novel subfamily of G-protein coupled receptors with 7 transmembrane domains. It could be activated by serine proteases such as mast cell tryptase or trypsin, for example. Therefore, the aim of present study was to investigate the effects of PAR-2 stimulation at some of neutrophils functions and activation. FACS data demonstrate increased levels of L-selectin shedding from the surface of human neutrophils after PAR-2 stimulation with a maximum at 6h after stimulation. Adding 10mM EDTA to the culture of stimulated human neutrophils abolished this effect. The increasing of L-selectin shedding could be one of the possible explanations the effects of PAR-2 stimulation on transendothelial migration (TME) ability of neutrophils. Our TME assay results show a decrease of neutrophil migration after PAR-2 stimulation (about 40%) as compared to unstimulated control. Furthermore, our ELISA data also demonstrate that PAR-2 agonists upregulate the release of IL-6 and IL-8 by human neutrophils. Taken together, these data allow us to suggest that PAR-2 plays a very important role in the regulation of neutrophil functions. Thus, PAR-2 may also play an important role in the pathophysiology of diseases associated with neutrophil disfunction such as psoriasis, bacterial infection, vasculatis, etc.

P063

Dermal dendritic cells differ in some aspects from Langerhans cells and may have a diffferent role in the immune response to a pathogen.

P. Stoitzner[1], C. Tripp[1], A. D. McLellan[2], N. Romani[1]

[1]Department of Dermatology, University of Innsbruck, 6020 Innsbruck, Austria
[2]Hematology Research Group, Christchurch School of Medicine, 12653 Christchurch , New Zealand.

Dermal dendritic cells differ in some aspects from Langerhans cells and may have a diffferent role in the immune response to a pathogen. It is well known that dermal dendritic cells and Langerhans cells generated from CD34+ precursor cells differ in phenotype and function. However, if there are the same differences between the Langerhans and dermal dendritic cells derived from skin has not been investigated. We isolated Langerhans cells after trypsinization of the epidermis and dermal dendritic cells after digestion with collagenase P. The cells were cultured up to 3 days with GM-CSF and used for phenotypical and functional studies. Dermal cell suspension were composed of an average of 6% dermal dendritic cells, around 3% T cells, under 1 percent macrophages, and the rest were fibroblasts. Dermal dendritic cells expressed MHC class II and CD11b, partly DEC-205 and CD40 and CD86, and are negative for CD11a and CD11c. So they differ from Langerhans cells in that they are of a 'more' mature phenotype and express lower levels of DEC-205. A subpopulation of Langerin-positive cells was found in the dermal cell populations, possible Langerhans cell precursors which increased in number during a 3 days culture. Both cell populations can be enriched by centrifugation on a Nycodenz gradient. Functionally, we found no difference in T cell stimulatory capacity between these two skin populations . Further investigations are under way.

P064

Alanyl aminopeptidase (APN/CD13) is involved in regulation of DNA synthesis on human keratinocytes and HaCaT keratinocytes *in vitro*

A. Thielitz[1,2], R. Vetter[1], U. Lendeckel[3], S. Ansorge[4], H. Gollnick[1], D. Reinhold[2]

[1]Otto-von-Guericke Universitaet, Klinik fuer Dermatologie und Venerologie, 39120 Magdeburg, Deutschland
[2]Institut fuer Immunologie, 39120 Magdeburg, Deutschland
[3]Institut fuer Experimentelle Innere Medizin, 39120 Magdeburg, Deutschland
[4]IMTM, 39120 Magdeburg, Deutschland

Alanyl aminopeptidase (APN/CD13) is a transmembrane ectoenzyme expressed on a wide variety of mammalian cells such as endothelial cells, cells of the myelo-monocytic lineage and fibroblasts. It is involved in the regulation of growth and function of activated T-cells *in vitro*. *In vivo*, constitutive expression on keratinocytes of patients with hyperproliferative skin diseases such as psoriasis, mycosis fungoides or lichen planus has been reported.The aim of the present study was to demonstrate *in vitro* the presence of CD13 on HaCaT- and primary keratinocytes and to answer the question if the proliferation of these cells can be influenced by inhibition of the enzymatic activity of APN.The expression of APN was investigated by RT-PCR and enzymatic activity assays which were performed in presence or absence of the specific APN inhibitors Actinonin and Bestatin. Proliferation was measured by ^{3}H-Thymidine-incorporation after 6 h incubation ± inhibitors. APN-mRNA could be detected in both primary and HaCaT keratinocytes. Both cell types show high specific enzymatic activity with 92 ± 15 pkat / 10^6 cells on HaCaT- and 160 ± 13 pkat / 10^6 cells on primary keratinocytes. A significant dose-dependent inhibition of DNA synthesis and enzymatic activity could be demonstrated after incubation with the above mentioned inhibitors on both cell types. Our data demonstrate that APN/CD13 is expressed in human keratinocytes and is involved in the regulation of DNA-synthesis of these cells *in vitro*. The specific enzymatic activity seems to be required for mediating this effect. This work was supported by the BMBF grant No.03WKD02H (Pharma MD).

P065

Comparison of Three Distinct Myeloid Dendritic Cell Subsets with Special Regard to Their Clinical Application in Melanoma Patients

G. Ratzinger[1,2], J. Reagan[1], M. Cos de Cossia[1], J. W. Young[1]

[1]Memorial Sloan Kettering Cancer Center, 10021 New York, NY, USA
[2]Univ.-Klinik für Dermatologie und Venerologie, 6020 Innsbruck, Oesterreich

Tumor immunity is not effectively induced in tumor-bearing hosts. This may be due to the lack of antigen-presentation by dendritic cells (DC) in malignant diseases. Several clinical trials have already shown that the administration of autologous, ex vivo generated DC loaded with synthetic tumor peptides can induce therapeutic immunity in Stage IV melanoma patients. But there is still a lot of parameters that have to be addressed to improve the efficacy of DC in clinical settings. We focussed on the comparison of three different subtypes of myeloid DC. We distinguish monocyte-derived dendritic cells (moDC), generated from blood-monocytes, Langerhans cell-like DC (LC) and dermal dendritic cell-like DC (DDC), both generated from CD34+ hematopoietic stem cells.
Phenotypical comparison showed LC to be positive for e-cadherin and negative for CD11b, moDC and DDC displayed an opposite expression pattern. Additionally moDC express high levels of CD91, the common heat shock protein receptor, and CD52, a molecule with unknown function that serves as target for the monoclonal antibody Campath-1H, already widely used in hematological disease. LC and DDC do not express CD52 at all. Looking at cytokine production we found differences concerning the production of IL-12, an important cytokine in the induction of TH1 immune responses. In response to either CD40L or an inflammatory cytokine cocktail moDC produce high levels of IL-12p70 while LC and DDC do not show any expression of this cytokine. We also investigated the uptake of apoptotic tumor cells by the different subsets of myeloid dendritic cells. We found that moDC take up huge amounts of apoptotic bodies within 24 hours, while LC and DDC show modest, but still substantial apouptake. The comparison of the stimulatory capacity in mixed leukocyte reactions revealed higher potency of LC and DDC to stimulate allogeneic T cells compared to moDC. Most clinical trials using dendritic cells have been performed with moDC. To further investigate a potential superiority of LC concerning their stimulatory capacity and thus their potency to elicit therapeutic immunity we are performing a clinical trial to compare tumor-antigen loaded moDC versus LC in Stage III and IV melanoma patients.

P066

The Autoantigen of Anti-p200 Pemphigoid Is a Non-collagenous N-linked Glycoprotein of the Dermal-Epidermal Junction

I. Shimanovich[1], E. Butt-Doerje[2], C. Sitaru[1], E. Broecker[1], Y. Hirako[1], D. Zillikens[1]

[1]University of Wuerzburg, Department of Dermatology, D-97080 Wuerzburg
[2]University of Wuerzburg, Institute for Clinical Biochemistry and Pathobiochemistry, 97080 Wuerzburg, Deutschland

Anti-p200 pemphigoid is an autoimmune subepidermal blistering disease characterized by autoantibodies to a 200 kDa protein (p200) of the dermal-epidermal junction (DEJ). The autoantigen localizes to the lower lamina lucida and, by indirect immunofluorescence microscopy on NaCl-split skin, the patients´ autoantibodies label the dermal side. Though p200 appears to be important for cell-matrix adhesion, the autoantigen has not yet been well characterized. Attempts to purify the protein from the dermis have been hindered by its insolubility in non-urea buffers. Nevertheless, p200 has been demonstrated to be different from other DEJ autoantigens, including bullous pemphigoid antigens 180 and 230, α6β4 integrin, laminin 5, and type VII collagen. The aim of the present study was the further biochemical characterization of p200. Differential extraction experiments demonstrated that efficient recovery of p200 from the dermis was strongly dependent on the presence of reducing agents, indicating that p200 is extensively cross-linked to other extracellular matrix components. In addition, p200 was resistant to digestion with bacterial collagenase, whereas this treatment did degrade major collagenous proteins of the dermis, including type I, VI, and VII collagen. This finding firmly established the non-collagenous nature of the p200 autoantigen. Treatment of dermal extracts with N-glycosidase F reduced the molecular size of p200 from 200 to 185 kDa without decreasing its immunoreactivity. These data suggest that N-glycosidically linked carbohydrates are present on the p200 molecule and that their removal does not affect epitope recognition by p200-specific autoantibodies. In contrast, digestion of p200 with neuraminidase, O-glycosidase, chondroitinase ABC, and heparinase III had no effect on its electrophoretic mobility, indicating that O-linked sugars and glycan side-chains are absent from the polypeptide backbone of p200. In summary, our data demonstrate that p200 is a non-collagenous N-linked glycoprotein of the DEJ. Further studies will be aimed at characterizing the role of this novel autoantigen in cell-matrix adhesion.

P067

Suppression of DC function following adenoviral transduction can be prevented by the use of gutless adenoviral vectors: Implications for immunotherapy using genetically modified DC

A. Tuettenberg[1], H. Jonuleit[1], T. Tueting[1], J. Brueck[1], V. Biermann[2], S. Kochanek[2], J. Knop[1], A. Enk[1]

[1]Johannes Gutenberg Universität Mainz, Hautklinik, 55131 Mainz, Deutschland
[2]Zentrum Molekulare Medizin Köln, 50931 Köln, Deutschland

Long-lasting and high-level gene expression in the absence of a toxic or inflammatory response to viral functions is necessary for the successfull application of genetically modified DC. We could previously demonstrate, that efficient transduction of mature $CD83^+$ DC using recombinant, replication-deficient ΔE1ΔE3 adenovirus suppressed their stimulatory capacity for $CD4^+$ and $CD8^+$ T cells. This process is not accompanied by alterations in the cytokine profiles induced or by changes of the T cell phenotype, it is independent of *de novo* synthesis of adenoviral proteins or NO production of infected DC, and is characterized by a cell cycle arrest of the stimulated T cells. In order to identify the viral structures responsible for the immunosuppression, we performed experiments with gutless Ad-vectors. Herein, 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. In our studies, we compared the transduction of DC using either ΔE1ΔE3 or gutless Ad-vectors. We demonstrate, that gutless Ad-vectors are suitable for an efficient transduction of mature human DC without an affection of morphology, phenotype and cytokine profile of the transduced DC population. Most importantly, using gutless vectors for transduction, DC showed a high allostimulatory capacity for $CD4^+$ and $CD8^+$ T cells. Thus, the impaired stimulatory capacity of ΔE1ΔE3 Ad-transduced, mature DC seems to be the result of an interaction of DC with adenoviral products that can be prevented using gutless Ad-vectors for transduction. These results have important implications for the use of genetically modified DC for therapeutic application.

P068

Production of interleukin 13 by human dendritic cells after stimulation with protein-allergens is a key factor for induction of Th2 cytokines and is associated with activation of STAT6

I. Böttcher[1], I. Bellinghausen[1], P. Brand[1], B. Klostermann[1], J. Knop[1], J. Saloga[1]

[1]University of Mainz, Department of Dermatology, 55131 Mainz, Deutschland

Dendritic cells (DC) are able to induce not only Th1 but also Th2 immune responses after stimulation with allergens. While DC-derived IL-12 and IL-18 are the key factors for the induction of Th1 cells, early signals being involved in Th2 differentiation are less well characterized so far. To analyze such early signals we used an antigen-specific setting with $CD4^+$T cells from atopic donors stimulated in the presence of autologous mature DC, which were pulsed with different allergen doses. The addition of increasing amounts of allergen during DC maturation with TNF-α, IL-1β and prostaglandin E2 resulted in enhanced secretion of IL-6 and IL-12 by DC followed by increased production of Th1 (IFN-γ) as well as Th2 (IL-4, IL-5) cytokines by $CD4^+$ T cells. The coculture of allergen-treated DC and $CD4^+$ T cells also led to a dose-dependent expression of active STAT6 which was visible already after 1 hour. Additionally, rapid phosphorylation of STAT6 was seen in immature DC after stimulation with allergens but not with LPS or HSA. STAT6 phosphorylation was associated with the production of IL-13 by DC. The addition of neutralizing anti-IL-13 antibodies during maturation of DC inhibited STAT6 phosphorylation in $CD4^+$ T cells as well as the production of IL-4 and to a lesser extend of IL-5, while IFN-γ production was not affected. Addition of exogenous IL-13 enhanced mainly the secretion of IL-4. Taken together, DC-derived IL-13 which is released after exposure to allergens appears to be one of the critical factors for DC to aquire the capability to induce Th2 cytokine production.

P069

Dendritic Cell Maturation and Differentiation by Osteopontin

A. C. Renkl[1], J. Wussler[1], T. Ahrens[2], K. Thoma[1], C. Bernardi[1], J. C. Simon[1], J. M. Weiss[3,1]

[1]Universitätsklinik Freiburg, Dermatologie und Allergologie, D-79104 Freiburg
[2]Universität Basel, Biozentrum, 4056 Basel, Schweiz
[3]Universitätsklinik Ulm, Dermatologie und Allergologie, D-89081 Ulm

Immature dendritic cells (DC) are located at epithelial borders. After activation and antigen uptake they mature and migrate into secondary lymphatic organs to initiate T-cell mediated immunity. As DC1 (high secretion of IL-12) or DC2 they are able to polarize naive T-cells towards either a Th1 or Th2 phenotype, decisively affecting the outcome of an immune response. Recently we demonstrated that Osteopontin (OPN) is important in cutaneous contact hypersensitivity by guiding DC into lymph nodes. Since OPN has been identified to have Th1 cytokine like properties, we now explored the effect of OPN on the phenotypic and functional maturation of DC. Human monocyte derived DC were cultured +/- OPN and supernatants and cells were analysed after 24 and 48h by ELISA or FACS. OPN strongly induced DC activation, upregulating their expression of HLA-DR, CD40, CD80, CD86, CD44 and CD56 and their TNF-α secretion. In allogeneic mixed lymphocyte reactions (MLR) OPN was either added directly into MLR with immature DC or OPN prestimulated DC were used. When OPN was added to MLR with immature DC, T-cell proliferation was enhanced, while addition of OPN to T-cells alone had no effect on their proliferation or cytokine secretion. However, when MLR was performed with OPN activated DC a strongly increased T-cell proliferation was detected compared to control DC. In MLR supernatants we found that both addition of OPN to MLR or OPN prestimulation of DC induced an upregulated secretion of the Th1 cytokine IFN-γ. Furthermore, high amounts of IL-12p70 were detected when OPN was added to the MLR, while IL-10 was not modulated. Coculture of naive Th cells (CD4+CD45RA+) with OPN matured DC resulted in an increased IFN-γ and a decreased IL-4 secretion, reflecting their Th1 polarization. In summary, our findings indicate that OPN induces DC maturation and their polarization towards a DC1 phenotype, indicating that the Th1 cytokine like properties of OPN are at least in part mediated through its effect on DC.

P070

Prevention of drug-induced antinuclear autoantibody formation by the adoptive transfer of previously exposed $CD4^+CD25^+$ regulatory T cells

L. E. Layland[1], M. Wulferink[1], S. Dierkes[1], E. Gleichmann[1]

[1]IUF, 40225 Duesseldorf, Deutschland

Drug-induced autoimmunity is often observed after the administration of certain drugs such as procainamide, mercury and gold. Although these xenobiotic compounds are different in structure and, perhaps, mechanistic action, the adverse immune reactions to these drugs share the development of antinuclear autoantibodies and the intricate involvement of $CD4^+$ T cells.
Our research focuses upon the involvement of T cells in procainamide-induced autoimmunity. We have found that after long-term oral treatment, $CD4^+$ T cells derived from treated A/J mice but not age-related controls, could respond *ex vivo* to neoantigens from procainamide-pulsed macrophages or the reactive metabolite of procainamide, *N*-hydroxylamine-PA. Here, we report that adoptive transfer of the $CD4^+CD25^-$ T cell sub-population from procainamide-treated donors into syngenic recipients, that were or were not already treated with procainamide for one week, initiated the development of autoantibodies. In contrast, the transfer of regulatory $CD4^+CD25^+$ T cells were able to prevent the autoantibody development in treated or untreated recipients. Therefore, to observe whether this suppressive phenomena could occur with other drugs, similar experiments using $HgCl_2$ and gold sodium thiomalate (GST) have been investigated. After several weeks of $HgCl_2$ treatment, autoantibodies developed in the donor C57BL/6 mice but not the controls. Following adoptive transfer into recipients also under $HgCl_2$ treatment, all recipient groups developed autoantibodies, but those injected with the regulatory $CD4^+CD25^+$ T cells from $HgCl_2$-treated donors were considerably reduced. Recipients not injected with $HgCl_2$ also developed autoantibodies but only when injected with the $CD4^+CD25^+$ T cells from $HgCl_2$-treated donors. Experiments using GST are continuing.
In conclusion, $CD4^+CD25^+$ T cells, previously exposed to a certain drugs, are able to hinder the development of autoantibodies in recipients undergoing treatment with the same drug. To our knowledge, this is the first time in which $CD4^+CD25^+$ T cells have been reported to play a role in drug-induced autoimmune diseases.

P071

In senescence decline of Th2 response can lead to Th1 response and resistance in *L.major* infected BALB/c mice

C. Sunderkötter[1,2], J. Ehrchen[1,2], E. Nattkemper[2], C. Sorg[2], K. Scharffetter-Kochanek[3]

[1]UK Münster, Hautklinik, 48149 Münster, Deutschland
[2]UK Münster, Inst. für Exp. Dermatol., 48149 Münster, Deutschland
[3]Univ. Ulm, Hautklinik, 89081 Ulm, Deutschland

Ageing is associated with a general decline in immunocompetence resulting in increased susceptibility to infectious agents. The senescent immune system shows e.g. dysregulation in production of NO or oxygen radicals by macrophages, and deficits of T cells with a decreased ratio of naive to memory T cells. In addition, ageing humans and mice may become less efficient in mounting an antigen-specific Th2 response. We wondered if in experimental leishmaniasis a decreased ability to elaborate Th2 cells would lead to a less severe course of infection in aged BALB/c mice, or if alterations of macrophage functions would lead to an aggravated course in resistant C57Bl/6 mice. When comparing bone marrow-derived macrophages from BALB/c or C57Bl/6 mice there was no significant difference in NO production and phagocytosis or killing of *L.major* between 2-month old and 18-month old (=senescent) mice.After infection of C57Bl/6 mice *in vivo* aged mice, like young mice, showed a resistant course without fatal dissemination. However, in BALB/c mice swelling and ulceration was less severe in aged mice and, surprisingly, in 6 of 8 aged mice ulcers healed. These mice also showed low or absent dissemination of *L.major* into visceral organs, and their CD4+ T cells released Th1 instead of Th2 cytokines in response to *L.major* antigen. When looking at possible reasons for this reversed immune reaction we found that macrophages from aged, but not from young BALB/c mice revealed constitutive release of low amounts of IL12, while there was no age-related difference after activation. Also, when ageing mice were kept in conditions free of specific pathogens (SPF) they did reveal decreased parasite load after infection, but no more Th1-determined resistance. We conclude i) that in ageing mice a decreased ability to mount a Th2 response can lead to emergence of a Th1 response and to resistance in BALB/c mice, while the immune response in C57Bl/6 mice remains unharmed, ii) that constitutive release of IL12 in ageing mice may favour the decline of Th2 response, and iii) that for full reversal into a Th1 response during ageing the immune system may need to be continuously stimulated, e.g. by exposure to microbes.

P072

Monitoring Humoral Responses to Human Melanocyte/Melanoma Differentiation Antigens - Establishing Capture ELISA Systems Specific for Tyrosinase-Related Protein (TRP)-1 and TRP-2

D. Thomas[1], W. Fink[1], G. Moldenhauer[2], D. Schadendorf[1], A. Paschen[1]

[1]Skin Cancer Unit of the German Cancer Research Center (DKFZ) at the University Hospital in Mannheim, 68135 Mannheim, Deutschland
[2]Department of Molecular Immunology at the DKFZ in Heidelberg, 69120 Heidelberg, Deutschland

Melanocyte/melanoma differentiation antigens (MDA), e.g. TRP-1 and TRP-2, have been clearly identified as molecular targets of immune responses in patients with vitiligo and malignant melanoma. Immunity against MDA is of diverse nature and includes T cell and B cell reactivity. Some studies indicate that antibodies might have an effector function although their occurrence might also reflect a secondary immunological event induced after melanocyte/melanoma cell destruction by antibody independent mechanisms. Regardless of their specific function, MDA-restricted antibodies indicate an ongoing immune response against cells of the melanocytic lineage and can be used as an indicator for the effectiveness of immunization protocols applied to melanoma patients. In order to examine humoral responses to TRP-1 and TRP-2 we set up a capture enzyme linked immunosorbent assay (ELISA). Therefore both human antigens were overexpressed as recombinant double-tagged proteins in *E. coli*, with a His-tag fused to the N-terminus and a myc-tag fused to the C-terminus of each protein. Recombinant proteins from crude bacteria lysate were directly immobilized on Ni^{2+} chelate-coated ELISA microwell plates. Binding of full-length recombinant protein was verified with an anti-myc monoclonal antibody indicating that purification of MDA antigens is not a prerequisite. Both ELISA systems are now employed to analyze sera from vitiligo and melanoma patients at different stages of the disease and with different medical histories for TRP-1 and TRP-2 specific antibodies. In addition, the assays will be used to determine MDA-restricted humoral responses in sera from mice treated with new experimental strategies against malignant melanoma.

P073

Induction of antigen-specific immunity in CDK4(R24C) knock-in C57BL/6 mice

J. Lenz[1], J. Steitz[1], S. Montag[1], G. Reinhard[1], S. Buechs[1], T. Woelfel[2], M. Malumbres[2], M. Barbacid[3], T. Tueting[1]

[1]Klinik für Dermatologie der Universität Bonn, 53105 Bonn, Deutschland
[2]3. Medizinische Klinik der Universität Mainz, 55131 Mainz, Deutschland
[3]Centro National de Investigaciones Oncologicas, 12546 Madrid, Spanien

C57BL/6 mice harboring an oncogenic R24C mutation in the cyclin dependent kinase 4 (cdk4) are of particular interest for the evaluation of novel antigen-specific melanoma vaccines because they are susceptible to in situ melanoma development after carcinogen treatment. In the present study we evaluated the ability to induce antigen-specific immunity against the model foreign antigen b-galactosidase (bgal) and the model melanocytic self-antigen Tyrosinase-related protein 2 (TRP2) in cdk4(R24C) knock-in C57BL/6 mice. Using ELISPOT- and ELISA-based assays, cellular as well as humoral immunity was demonstrated both in wild type and in cdk4(R24C) knock in C57BL/6 mice following injection of recombinant adenoviruses encoding bgal or human TRP2. Furthermore, gene gun immunization targeting the skin of mice with an expression plasmid encoding human TRP2 resulted in coat depigmentation as a sign of autoimmune-mediated destruction of melanocytes. In conclusion, cdk4(R24C) knock-in C57BL/6 mice appear capable of mounting antigen-specific immune responses. Future studies will have to show whether carcinogen treatment adversely affects the efficacy of antigen-specific immunization and whether the induction of TRP2-specific immunity can eradicate carcinogen-induced neoplastic melanocytic lesions.

P074

Genetic immunization targeting the skin with the gene gun: Comparative assessment of plasmid DNA and synthetic RNA

G. Reinhard[1], J. Steitz[1], J. Lenz[1], S. Montag[1], S. Buechs[1], P. Speuser[1], C. Britten[2], T. Woelfel[2], T. Tueting[1]

[1]Klinik für Dermatologie der Universität Bonn, 53105 Bonn, Deutschland
[2]3. Medizinische Klinik der Universität Mainz, 55131 Mainz, Deutschland

Bombardment of the skin with expression plasmids precipitated onto gold particles using the gene gun leads to the induction of strong antigen-specific cellular and humoral immunity to the encoded antigen. Recently, genetic immunization has also been performed with messenger RNA as an alternative immunogen. The use of RNA avoids the risk of mutagenic insertion into the genome but is associated with rapid degradation and limited antigen expression. In the present study we investigated the efficacy of gene gun immunization using synthetic RNA in direct comparison with plasmid DNA. Initially, our studies focussed on enhanced green fluorescent protein (EGFP) and β-galactosidase (βgal) as model antigens in C57BL/6 mice. EGFP- and βgal-encoding RNA was transcribed in vitro from expression plasmids and subsequently polyadenylated. Mice were shot into the shaved abdominal skin at a pressure of 400 psi using the gene gun resulting in the delivery of 15µg RNA precipitated onto 1 mg of gold beads. Control mice received 1µg plasmid DNA. Using X-gal staining and fluorescence microscopy on skin sections harvested 24h later, antigen expression by in vivo RNA-transfected keratinocytes could be verified. Importantly, ELISPOT analyses demonstrated the induction of antigen-specific T cells recognizing the H-2Kb-binding peptide ICPMYARV derived from amino acid 497-504 of β-galactosidase following genetic immunization with synthetic RNA. Furthermore, antibodies specific for recombinant β-galactosidase protein could be detected in serum harvested from RNA-immunized mice using a sandwich ELISA. In direct comparison with plasmid DNA, the amount of antigen expressed in the skin and the magnitude of the specific immune response appeared to be slightly reduced in mice immunized with RNA. In conclusion, gene gun immunization with synthetic RNA appeared almost as effective as plasmid DNA. Eventually, this method will be applied to investigate the induction of cellular immune responses against the murine melanoma antigen TRP-2.

P075

Construction of fusion proteins between a melanocytic self-antigen and an immunogenic foreign antigen in order to break immune tolerance: The intracellular localization does not influence immunogenicity

J. Brueck[1], J. Steitz[1], J. Lenz[1], T. Tueting[1]

[1]Klinik fuer Dermatologie der Universitaet Bonn, 53105 Bonn, Deutschland

Previously, we reported that gene gun immunization of C57BL/6 mice with plasmid DNA encoding a fusion protein between the autologous, murine melanosomal enzyme tyrosinase-related protein 2 (TRP2) and the immunogenic enhanced green fluorescent protein (TRP2-EGFP) was able to break immune tolerance and stimulate CD8+ T-cells in vivo recognizing the H2-Kb-binding peptide TRP2aa180-188. TRP2 is a glycosylated type I transmembrane protein with a N-terminal signal sequence for translation into the ER (~aa1-24), a luminal enzymatic domain(~aa25-470), a transmembrane domain (~aa471-495) and a C-terminal cytoplamatic tail (~aa496-517) containing trafficking signals for melanosomes. Fusion constructs with immunogenic proteins considerably perturb the intracellular localisation of TRP2 and might alter the immunogenicity. Therefore, we constructed various fusion proteins between murine TRP2 and EGFP with different intracellular localisation by (1) inserting EGFP into the luminal domain, (2) replacing the C-terminal transmembrane and cytoplasmatic domain of TRP2 with EGFP, (3) additionally removing the ER signal sequence, and (4) replacing the ER signal sequence by amino acid 1-80 of murine invariant chain. The various constructs were stably introduced in the murine cell line DCEK and the intracellular localisation analyzed by confocal laser microscopy. Subsequently, genetic immunization of C57BL/6 mice was performed with all constructs using the gene gun. Induction of TRP2-specific CD8+ T-cells in vivo and autoimmune-mediated destruction of melanocytes in the bombarded area was observed in every case. In conclusion, the nature of the fusion protein and the intracellular localisation did not influence the resulting immune response in this model system.

P076

6-Sulfo LacNAc, a Novel Carbohydrate Modification of PSGL-1, Defines an Inflammatory Type of Human Blood Dendritic Cells

K. Schäkel[1,2], R. Kannagi[3], B. Kniep[1], Y. Goto[3], C. Mitsuoka[3], J. Zwirner[4], A. Soruri[4], M. von Kietzell[1], E. P. Rieber[1]

[1]Institute of Immunology, Medical Faculty, 01307 Dresden, Deutschland
[2]Department of Dermatology, Medical Faculty, Technical University of Dresden, 01307 Dresden, Deutschland
[3]Program of Experimental Pathology, Aichi Cancer Center, 01307 Nagoya, Japan
[4]Department of Immunology, University of Göttingen, 01307 Göttingen, Deutschland

Dendritic cell (DC) subsets differ in their tissue distribution, their response to pathogens and their ability to polarize T cells. In human blood the mAb M-DC8 and two mAbs (DD1 and DD2) generated in addition, define a distinct population of blood cells with features of immature blood dendritic cells (DCs). In a westernblot analysis of membrane molecules prepared from M-DC8+ cells the mAb M-DC8 and two novel mAbs (DD1 and DD2) showed a reactivity with dispersed bands of 120 kD and 240 kD. Sequential immunoprecipitation revealed that the M-DC8-structure is present on the P-selectin glycoprotein ligand 1 (PSGL-1). Further characterization of the M-DC8-structure using enzymatic carbohydrate degradation, inhibition of carbohydrate neo-genesis and cDNA-transfection revealed that the mAb M-DC8 defines a sulfated, non-sialylated and O-linked carbohydrate moiety. Screening of synthetic glycolipids identified the M-DC8-structure as 6-sulfo LacNAc. In contrast to previously described blood DCs (DC1 and DC2), circulating in blood at a much lower frequency (M-DC8+ DCs 1,2%; DC1 0,6%; DC2 0,2%; n=20), M-DC8+ DCs lacked the cutaneous lymphocyte antigen on PSGL-1 and failed to bind P- and E-selectin-constructs. Yet, M-DC8+ DCs cells expressed anaphylatoxin receptors (C5aR, C3aR) and FcgRIII (CD16), known to facilitate rapid recruitment to inflammatory cites. Indeed, C5a injected into the intraperitoneal cavity of SCID mice stimulated the immigration of M-DC8+ DCs. Upon direct comparison, M-DC8+ cells were as potent as DC1 in inducing the proliferation allogeneic cord blood T cells and priming of autologous T cells for KLH. In accord with an inflammatory cell type M-DC8+ DCs produced by far more TNF-α upon stimulation with LPS than DC1 and monocytes. Taken together, we identified the mAb M-DC8-epitope as 6-sulfo LacNAc, an exclusive modification of PSGL-1 on the surface of the majority of human blood DCs which have a pro-inflammatory capacity.

P077

Nickel (Ni) tolerance in mice: a joint venture of APCs, T suppressor cells, NKT cells, and apoptosis

K. Haarhuis[1], X. Wu[1], M. Nowak[1], F. Kopp[1], S. Artik[1], E. Gleichmann[1]

[1]Institut fuer Umweltmedizinische Forschung, 40225 Duesseldorf, Deutschland

Previous studies from our laboratory showed that $NiCl_2$ lack intrinsic adjuvanticity and that they need to be administered together with adjuvant in order to induce *de novo* sensitization. Not surprisingly, therefore, tolerance to Ni can be induced by oral administration of $NiCl_2$. However, mouse strains lacking either Fas, FasL, or NKT cells proved resistant to oral tolerance induction to Ni, indicating that Fas-mediated apoptosis and NKT cells play a role in this model. The T cells of orally tolerized B6 mice proved to be anergic and suppressive, and their splenic APCs showed a reduced allo-stimulatory capacity and displayed an immature phenotype. Adoptive transfer of as few as 10^2 splenic T cells or 10^2 splenic APCs from tolerant donors prevented *de novo* sensitization to Ni of the recipients. Unlike their T cells, the APCs of Ni-tolerant donors lost their capacity to transfer the tolerance 20 wks after termination of oral $NiCl_2$ treatment.
Considering the extremely low cell number capable of adoptively transferring Ni tolerance, we asked whether infectious tolerance is at play here. The term infectious tolerance is used to denote the phenomenon that in serial adoptive cell transfer experiment tolerance is infectiously spread from tolerant donor T cells to the T cells of the recipient. Recipient T cells thus infected do not only become tolerant but also acquire the infectious capacity of the tolerant T cells from the original donor in that they can further transfer the tolerance to a new set of recipient mice. We showed that, indeed, an infectious spread of Ni tolerance from donor T cells to host T cells takes place upon adoptive transfer. However, this infectious spread of tolerance requires participation of the recipient's APCs. The tolerant, Ni-specific donor T cells apparently modify Ni-presenting APCs of the recipient into tolerogenic APCs, which in turn can induce tolerance in naive T cells. Interestingly, in order to become effective upon adoptive transfer, the spread of tolerance to the recipient requires not only Ni presentation but also costimulation, indicating that the Ni-specific Ts cells involved here need to be fully re-activated. The combined findings show that tolerant T cells can modify APCs and *vice versa*, this amplification ot toleance effectively abrogating unwanted activation of Ni-specific effect

P078

Tumor immune escape by loss of chemokine expression

J. Rieker[1], A. Mueller[2], R. C. Kubitza[1], T. Ruzicka[1], A. Zlotnik[3], B. Homey[1]

[1]Heinrich-Heine University, Department of Dermatology, 40225 Duesseldorf, Deutschland
[2]Heinrich-Heine University, Department of Radiation Oncology, 40225 Duesseldorf, Deutschland
[3]Eos Biotechnology, 94080 South San Francisco, USA

Recruitment of leukocytes to peripheral organs is a prerequisite to maintain immunosurveillance and sustain homeostasis. Chemokines have been shown to critically regulate leukocyte trafficking. Recently, we identified the novel skin-associated chemokine CCL27(CTACK, ALP, ESkine) and its receptor CCR10(GPR-2) which specifically mediate the recruitment of lymphocytes into the skin. In the present study, we focused on the role of CCL27 and CCR10 in skin homeostasis and nonmelanoma skin cancer. While CCL27 was most abundantly expressed in basal keratinocytes of the epidermis in normal skin, transformed keratinocytes of basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (SCC) showed loss of CCL27 protein expression. Moreover, also keratinocytes of actinic keratosis exhibited the absence of CCL27 expression indicating that loss of homeostatic chemokine expression may occur early in malignant transformation. Immunohistochemical results were confirmed by real-time quantitative PCR analyses showing marked expression of CCL27 mRNA in cultured primary keratinocytes whereas this chemokine was undetectable in SCC cell lines. The loss of CCL27 expression in BCC was accompanied by a disorganized distribution of peritumoral lymphocytes and macrophages. In addition, invasion of T cells into the tumor nests was hardly observed. Taken together, these findings suggest that the loss of chemokine expression by malignant cells may provide a novel mechanism for the evasion of anti-tumor immune responses.

P079

TNF-alpha induces semi-maturation of dendritic cells, retaining them flexible for the induction of T cell tolerance, Th 1 or Th 2 immunity

C. Voigtlaender[1], M. Menges[1], S. Roessner[1], G. Schuler[1], M. B. Lutz[1]

[1]Dermatologie der Universität Erlangen, 91052 Erlangen, Deutschland

We recently demonstrated that partial maturation of DC occurs upon TNF-α treatment. Such semi-mature DC could induce tolerance by inducing CD4+ IL-10+ regulatory T cells when injected i.v. into mice. Here we further characterized TNF/DC and investigated their immunomodulatory capacity in vivo. We found that the expression of MHC and costimulatory molecules did not differ between semi-mature TNF/DC and fully mature DC that were stimulated with LPS plus anti-CD40. Functionally, TNF/DC showed the same allogeneic T cell priming capacity in vitro than DC matured with LPS, but less than fully mature LPS/CD40/DC. TNF/DC do not produce IL-12p70 but were not exhausted, since they were able to produce IL-12 upon further treatment with LPS/anti-CD40. This underlines the functionally intermediate maturation stage of TNF/DC. In vivo we found - in contrast to the i.v. injections - that single s.c. injection of TNF/DC preferentially induced mixed Th2>Th1 responses whereas repetitive s.c. injections of the same DC lead to induction of a clear Th1 polarisation. In summary, TNF/DC can induce Th1, Th2 or regulatory T cells, depending on the route of administration and the number of injections. The mechanisms underlying the different polarisation of T cells after i.v. and s.c injections remain to be elucidated and are subject of further studies.

P080

Perforin (Perf) Reduction of All Lymphocytes and of the $CD8^+$ $CD28^-$ Subtype in Alopecia Areata (AA) is Independent of an Atopic Diathesis but Correlates with Disease Acitivity

I. Schmidt[1], A. Ambach[1,2], B. Bonnekoh[1], H. Gollnick[1]

[1]Otto-von-Guericke-University, Dep. Dermatology & Venereology, 39120 Magdeburg, Deutschland
[2]Otto-von-Guericke-University, Center for Immunological Research, 39120 Magdeburg, Deutschland

Different types of regulatory lymphocytes are assumed to exist: 1) $CD4^+$ $CD25^+$ cells secreting IL-10, 2) KIR^+ $CD3^+$ NKT cells recognizing lipid antigens, 3) $CD8^+$ $CD28^-$ T cells using Perf-granules to prevent B7-upregulation on APCs and, thereby, hindering T helper activation. $CD8^+$ cells play a key role in the pathogenesis of AA, and a defect of the Perf-system in AA was described by us recently. The contribution of $CD8^+$ $CD28^-$ cells to this defect is unclear. In addition, AA is associated in up to 50% with atopy, and the Perf-system in atopic patients was shown to be distorted. Therefore, we asked for a role of atopy in the Perf-defect of AA.

Ficoll-isolated peripheral mononuclear cells of 22 AA-patients (no systemic therapy; <30% scalp involvement n=15, ≥30% n=7) were stained with monoclonal antibodies against CD8, Perf, CD28, investigated by flow cytometry, and compared to 12 healthy controls (HC).

The percentage of $Perf^+$ lymphocytes in *atopic* (n=8, 19±6%, $p<0.01$) as well as in *non-atopic* AA-patients (n=14, 27±9%, $p<0.05$) was found to be reduced as compared to HC (37±12%). In patients with clinical signs of AA-activity (n=12), significant fewer $Perf^+$ lymphocytes (21±7%) were detected as compared to HC (37±12%, $p<0.01$). Patients without AA-activity showed less Perf-reduction (28±10%, $p=0.08$). No correlation was found between Perf-expression and extent of scalp involvement. Focusing on the contribution of $CD8^+$ $CD28^-$ lymphocytes to the Perf-reduction in AA: 70±18% of these cells stained $Perf^+$ in AA as compared to 82±9% in HC ($p<0.05$). This was mediated by $CD28^-$ lymphocytes expressing low levels of CD8 ($CD8^{dim+}$; AA 90±5%; HC 96±3%; $p<0.01$), but not by $CD8^{hi+}$ $CD28^-$ T cells (AA 56±23%; HC 60±20%; $p=0.63$). Reduced Perf-expression of $CD8^{dim+}$ $CD28^-$ cells was seen in *atopic* (89±7%, $p=0.01$) and *non-atopic* AA-patients (91±5%, $p<0.01$).

Taken together (i) Perf-reduction in AA cannot only be explained by patients with an atopic diathesis, (ii) Perf-reduction is pronounced in patients with active AA pointing to its pathophysiological relevance, (iii) $CD8^+$ $CD28^-$ lymphocytes contribute to the Perf-defect.

P081

UV-Injury, apoptosis, chemokines and leukocyte recruitment: an amplification cycle triggering cutaneous lupus erythematosus

S. Meller[1], R. Kubitza[1], H. Kanzler[2], M. Gillliet[3], U. Wiesner[1], M. Dieu-Nosjean[4], T. Ruzicka[1], P. Lehmann[5], A. Zlotnik[6], B. Homey[1]

[1]University of Duesseldorf, Department of Dermatology, 40225 Duesseldorf, Deutschland
[2]Dynavax Technologies, CA 94710-2 Berkeley, USA
[3]University or Zuerich, Department of Dermatology, CH-8091 Zuerich, Schweiz
[4]INSERM, Laboratoire dâ€™Immunologie Cellulaire et, 75270 Paris, Deutschland
[5]University of Witten-Herdecke, Department of Dermatology, 42117 Wuppertal, Deutschland
[6]Eos Biotechnology, 94080 South San Francisco, USA

Lupus erythematosus (LE) is an autoimmune disease which shows photosensitivity, increased apoptosis, and cutaneous leukocyte recruitment. In the present study, we identified that the CXCR3 ligands, CCL9 (Mig), CXCL10(IP-10), CXCL11(I-TAC) being the most abundantly expressed chemokine family members (n=42) in cutaneous LE. Expression of these ligands corresponded with a marked inflammatory infiltrate consisting of mainly CXCR3-positive cells including CLA+ lymphocytes and CD123+/BDCA-2+ plasmacytoid dendritic cells (pDC). Circulating pDCs expressed CCR5, CXCR3, and CXCR4 and produced high levels of type I interferon as well as chemokines after stimulation with either virus, apoptotic bodies or immunostimulatory DNA sequences (ISS). Type I interferons, in turn, were potent inducers of CXCR3 ligands in cellular constituents of the skin such as keratinocytes, dermal fibroblasts and endothelial cells. Furthermore, we could show that UVB-irradiation induces a distinct set of chemokines in keratinocytes including CCL5, CCL20, and CCL27. Taken together our data suggest an amplification cycle with UV-injury inducing apoptosis and chemokine production which in turn mediate the recruitment and activation of lymphocytes and IFN-α-producing pDCs which subsequently release more effector cytokines and chemokines finally leading to the development of cutaneous LE lesions.

P082

IFNγ induced ectopic MHC class I expression in human hair matrix cells is down-regulated by IGF-I, α MSH and TGFβ1: Implication for hair follicle immue privilege and alopecia areata

T. Ito[1], N. Ito[1], Y. Tokura[2], M. Takigawa[3], R. Paus[1]

[1]University Hospital Eppendorf, Hamburg, Dermatology, D-20249 Hamburg
[2]University of Occupatinal and Environmental Health, Dermatology, 807-8555 Kitakyushu, Japan
[3]Hamamatsu University School of Medicine, Dermatology, 431-3192 Hamamatsu, Japan

The anagen hair bulb displays an immune privilege, e.g. it has low or absent MHC class I expression on the protein level. Here we show by Abidin Biotin Complex, EnVision and Tyramide Signal Amplification technique that there are substantial differences in the consititutive follicular expression pattern of MHC class I between different individuals and different techniques. We confirm by in situ hybridization that there is lor or absent MHC class I mRNA extraction in the hair matrix. We have previously hypothesized that collapse of the proximal MHC class I negativity of hair follicle is a crucial element in the pathogenesis of alopecia areata (AA), an autoimmune disease, during which ectopic MHC class I expression in the hair bulb plays a critical role (Paus et al. Yale Biol Med 1994). Since we have previously shown in mice that IFNγ is the key factor of up-regulation of MHC class I in anagen hair bulb (Rueckert et al. JID 1998), we have tested whether this is also true in organ-cultured human anagen scalp hair follicles. We demonstrated that IFNγ (75U/ml) indeed strongly induces ectopic MHC class I expression in the hair matrix. Next, we studied for effective agents whcih can down-regulate this ectopic MHC class I expression in human hair follicle. Here we show immunohistochemistry that IGF-I (100ng/ml), αMSH (0.4μg/ml) and TGFβ1 (30ng/ml) significantly down-regulate the IFNγ induced ectopic MHC class I expression in normal human anagen hair bulb in situ. We also analyzed mRNA level of MHC class I expression by in situ hybridization. In addition, we have studied the effects of these agents on TAP2 and calnexin. This is the first report of down-regulators of ectopic MHC class I expression in human hair follicle. It suggests that these agents may be used to restore a collapsed hair follilce immune privilege, and may therefore be attractive new treatment for alopecia areata.

P083

BP180-and BP230-specific IgG autoantibodies as markers for disease activity and prognosis of bullous pemphigoid

S. Thoma-Uszynski[1], W. Uter[2], S. Schwietzke[1], S. C. Hofmann[1], L. Borradori[3], M. Hertl[1]

[1]Universität Erlangen-Nürnberg, Dermatologische Klinik, D-91052 Erlangen
[2]Universität Erlangen-Nürnberg, Abt. für Medizinische Informatik, Biometrie und Epidemiologie, 91054 Erlangen, Deutschland
[3]Universität Genf, Dermatologische Klinik, CH-1211 Genf, Schweiz

Bullous pemphigoid (BP) represents a model of antibody mediated autoimmunity. The hemidesmosomal adhesion molecule BP180 is of eminent importance for dermoepidermal adhesion and defined target for autoantibodies (auto-ab) in BP. The intracellular plaque component BP230 is also recognized by auto-ab, but their pathogenetic significance is unclear at present.This study was aimed to characterize auto-ab profiles by ELISA using recombinants of BP180 and BP230, in 148 BP patients.Within this cohort, 12 patients were in remission, 43 affected by localized and 93 by generalized disease. 51 age and sex matched individuals served as controls. We analyzed BP180- and BP230-specific auto-ab responses (IgG, IgG1, IgG4) with regard to age, sex, clinical state (remittent, localized, generalized) and disease duration. Among patients, there were 57.4% females with no significant differences between clinical categories. The median age was 78 years, whereby older patients were significantly more often generally affected. Regarding BP180, there was a highly significant association between diagnosis of BP and positive BP180 reactivity with 95.3% for any one of the clinical categories.Most patients displayed also BP230 reactivity: 127(88.8%) had simultaneous BP180 and BP230 reactivity. In addition, positive BP230 reactivity was significantly associated with localized BP, but not with other clinical states. The global diagnostic properties of BP230 were moderate compared to BP180 (sensitivity 0.885 vs. 0.953, specificity 0.684 vs. 0.940). BP180 IgG levels were significantly lower after a longer history of BP in the "generalized" group, and for localized disease regarding BP230 IgG.Correlation between total IgG and IgG1 and IgG4 was variable for both BP180 and BP230 with correlation coefficients ranging from 0.39 to 0.80.In summary, the global diagnostic properties of BP180 reactivity outrage those of BP230. BP230 reactivity, however, further supports the diagnosis of BP and might be indicative for a localized course of the disease.The established assays provide useful markers for prognosis and disease monitoring in BP.

P084

Intradermal genetic vaccination: Role of bone marrow-derived antigen presenting cells

A. Schneeberger[1], P. Luehrs[1], R. Kutil[1], C. Wagner[1], G. Stingl[1], S. N. Wagner[1]

[1]Univ. Vienna Medical School, DIAID, Dept. of Dermatol., 1090 Vienna, Oesterreich

We have recently shown that intradermal (i.d.) injection of pDNA coding for the autologous form of Pmel17 induces a specific T cell response capable of protecting DBA/2 mice against a challenge with a lethal dose of Pmel17-expressing melanoma cells. In the present study, we attempted to explore the mechanism(s) of this protective response.To this end, we used β-galactosidase (βgal) as a surrogate antigen. A single i.d. injection of a CMV-driven VR1012 vector engineered to express the lacz gene led to the activation of βgal-specific CD4+ and CD8+ T cells as well as antibodies. Excision of the VR1012 injection site at defined time points after vaccination (d1, 4, 6, 8, 11, 13) revealed that, for a specific immune response to occur, the i-site has to stay intact for 8 days. To test whether migratory antigen presenting cells (APC) or resident non-leukocytic cells are the critical sensitizing cell population, bone marrow chimeras were employed. F1 (C57BL/6 x BALB/c) mice that had been reconstituted with either BALB/c or F1 bone marrow were used 3 months after transplantation, i.e. at a time when >95% of migratory skin DC were of donor origin. Following i.d. application of 50μg VR1012lacz, BALB/c-reconstituted F1 were found to harbor T cells that recognized an H-2Ld-restricted peptide epitope but none that reacted against an H-2Kb epitope potentially presented by resident skin cells which express both H-2d and H-2b MHC molecules and recognized by the T cells that had been educated in an H-2d/b thymic microenvironment.Our results suggest that bone marrow-derived APC crosspresent proteins produced by activated skin cells (e.g., keratinocytes).

P085

Toll-like receptor 2- and 4-dependent regulation of epithelial inflammatory signaling

M. K. Oeff[1], H. Seltmann[1], N. Hiroi[2], A. Nastos[2], S. R. Bornstein[2], C. C. Zouboulis[1]

[1]University Medical Center Benjamin Franklin, Department of Dermatology, 12200 Berlin, Deutschland
[2]Heinrich Heine University, Department of Endocrinology, D-40225 Duesseldorf

Human epithelial cells are likely to modulate inflammatory and immune processes by intrinsic mechanisms. Toll-like receptor (TLR) which are - together with CD14 - involved in the innate immunity and are able to recognize microbial components. They have been shown to be expressed not only in inflammatory cells but also in human keratinocytes. After having detected the expression of CD14 in human sebocytes we addressed here the hypothesis of TLR expression in these cells after prolonged (+24 h) exposure and its modulation by components of gram-negative (LPS) and gram-positive (LTA) bacteria. Changes in TLR expression by these agents were compared to the action of retinoids, hydrocortisone and phorbol 12-myristate 13-acetate (PMA). The regulation of TLR mRNA levels was investigated by using the TaqMan quantitative PCR method. To detect the possible pro- or anti-inflammatory effects of microbial components and immunosuppressive drugs we measured the IL-8 expression in human SZ95 sebocytes by TaqMan quantitative PCR and its secretion by ELISA, respectively. SZ95 sebocytes expressed TLR2 and TLR4 on mRNA and protein levels, as detected by RT-PCR and immunocytochemistry. LPS enhanced TLR2 and IL-8 expression ($n=7$, $p<0.05$), while LTA barely regulated the expression of TLR2 and 4. While TLR2 expression could not be modified by hydrocortisone, the latter suppressed TLR4 and IL-8 expression ($n=7$, $p<0.05$). PMA increased IL-8 and TLR2 and 4 expression dramatically ($n=7$; $p<0.001$). Neither IL-8 nor TLR expression was regulated by retinoids. The present work provides first evidence that bacterial cell wall components may directly affect human sebocytes via a specific receptor-dependent mechanism in the absence of inflammatory cells.

P086

Systematic Optimization of Dendritic Cell Phenotype and Function before Use in Cancer Immunotherapy

H. A. Haenssle[1], T. Buhl[1], S. Bram[1], C. Neumann[1]

[1]Georg-August University Goettingen, Dermatology, D-37075 Goettingen

Dendritic cells (DCs) are probably the most potent cells of the immune system for activation and regulation of both cellular and humoral responses. It is well known that the function of DCs highly depends on their level of maturation. In this *in vitro*study we analysed and optimized six maturation protocols with regard to the resulting DC phenotype and function. The six protocols included maturation by CD-40 ligation, by unmethylated CpG-dinucleotides (CpG-motifs), by a cytokine cocktail (CC) consisting of IL-1β, IL-6, TNF-α and PGE2, by a combination of CC plus CD-40L, by a combination of CC plus CpG and by a combination of CC plus CD40L plus CpG. The DC-phenotype was evaluated by flourescence microscopy and flowcytometry of the following markers: HLA-DR, CD14, CD83, CD80, CD86, CD58 and CD40.In order to determine functional aspects of the generated DCs an allogenic 3[H]tymidine proliferation assay of T cells, a quantitative RT-PCR for several DC cytokines (IL-10, IL-12p35, IL-12p40, IL-16, IL-5, IFN-γ, Lymphotactin, TGF-β, TNF-α) and ELISPOT analysis of autologous lymphocytes, that were stimulated by tetanus toxoid (tt) loaded DCs were performed. **RESULTS:**Using a two-step culture model, we were able to detect significant differences in terms of DC phenotype, cytokine production and stimulation of allogeneic or autologous T-lymphocytes between the six maturation protocols analysed.The achieved level of phenotypical DC maturation closely correlates with the amount of allogenic T cell proliferation and the frequency of IFN-γ secreting tt-specific T-lymphocytes after stimulation with tt loaded DCs. The resulting DCs can be graded into three groups according to their grade of maturation and their T-cell stimulatory capacity:
LOW (immature DCs), **INTERMEDIATE** (CD40L, CpG) and **HIGH** (CC, CC plus CD40L, CC plus CpG, CC plus CD40L plus CpG).The amount of DC-I based cytokine mRNA as determined by quantitative-RT-PCR is highly dependent on the leukapheresis-donor and often does not match the above ranking.

P087

Stage-dependent downregulation of MHC class-II molecules and CD86/B7-2 on peripheral blood monocytes of melanoma patients.

S. Ugurel[1], D. Uhlig[2], W. Tilgen[2], U. Reinhold[2]

[1]Klinische Kooperationseinheit Dermato-Onkologie, Deutsches Krebsforschungszentrum Heidelberg und Universitätsklinikum Mannheim, 68135 Mannheim, Deutschland
[2]Universitäts-Hautklinik und Poliklinik, Universitätskliniken des Saarlandes, 66421 Homburg/Saar, Deutschland

Antigen presenting cells play a crucial role in the development and maintenance of an antigen-specific anti-tumoral immune response. Recently we could show a stage-dependent decrease of serum concentration of soluble MHC class-II molecules sHLA-DR in melanoma patients. In the present study we investigated peripheral blood monocytes (PBM) as a possible source of origin of this serum factor. The surface expression of MHC class-I and -II molecules, of the costimulatory molecules CD80/B7-1 and CD86/B7-2 and of the marker of proliferation CD71 were analyzed on CD14+ PBM from 144 melanoma patients and 43 healthy controls using flow cytometry. We found decreased expression of HLA-DR ($p < 0.0005$), HLA-DP ($p < 0.0005$), HLA-DQ ($p = 0.006$) and CD86/B7-2 ($p = 0.001$) on PBM from melanoma patients compared to healthy controls, associated to advanced stages of disease and tumor burden, whereas no significant differences could be detected in HLA class-I and CD80/B7-1 expression. In contrast, expression of CD71 was increased on PBM from melanoma patients compared to healthy controls ($p = 0.024$). The mode of therapy currently given to the patients analyzed showed no effect on the ecpression pattern of PBM. In conclusion, our results indicate the downregulation of HLA-DR expression on PBM as one possible source of origin for the reduced sHLA-DR found in sera from melanoma patients. Moreover, our findings suggest an impaired antigen-presenting function of PBM in melanoma patients which might impair immunotherapeutic strategies.

P088

Dendritic cells do not produce interferon-γ

A. Abdelhafez[1], U. Kaemmerer[2], A. D. McLellan[1], A. O. Eggert[1], C. Linden[1], M. Kapp[2], U. Bommhardt[3], E. Broecker[1], E. Kaempgen[1]

[1]Univ. Dep. Dermatology, 97080 Wuerzburg, Deutschland
[2]Univ. Dep. Gynecology, 97080 Wuerzburg, Deutschland
[3]Inst. Immunobiology, 97080 Wuerzburg, Deutschland

Recently there have been several reports on the production of IFN-γ by murine dendritic cells (DC) in response to stimulation with IL-12. These have been conflicting in the description of the subsets producing the IFN-γ and the cytokines synergizing with IL-12 for IFN-γ production. Here we present evidence that the source of IFN-γ in all these reports are T and/or NK-T cells. Spleen DC preparations sorted for high CD11c and MHCII expression only responded to IL-12 with IFN-γ secretion when costimulated with IL-2 and/or IL-18. Intracellular IFN-γ staining revealed that a small MHC class II- population contaminating these preparations was the source of IFN-γ. Flow cytometry also showed that few CD3+, Pan-NK-T or NK-T cells were responsible for IFN-γ production within cultures of GM-CSF/IL-4 generated bone marrow DC. Furthermore, a percentage of these cells stained for MHCII demonstrating that they are clustered to DC and therefore easily contaminate DC in MHCII and/or CD11c dependent DC isolation procedures. Human monocyte derived DC produced trace amounts of IFN-γ only under stimulation with combinations of IL-12, IL-2 and IL-18. Immunocytochemistry revealed that the cells responsible for the IFN-γ production were rare contaminating cells of lymphocytic morphology. In summary, thorough exclusion of contaminating cells in both the murine and human system consistently led to decreasing amounts of IFN-γ and increasing requirements for IL-2 and IL-18 costimulation, both established activators of T and NK-T cells. The overall pattern of the available data thus implies that the source of IFN-γ reported to be of DC origin in previous studies is always a small number of contaminating T and NK-T cells, that tightly adhere to DC and are difficult to exclude.

P089

Imiquimod treatment of cutaneous T cell lymphoma

A. Born[1], S. Gellrich[1], K. Kotsch[2], D. Volk[2], W. Sterry[1], J. Muche[1]

[1]Charité Berlin, Dermatologie, 10098 Berlin, Deutschland
[2]Charité Berlin, Immunologie, 10098 Berlin, Deutschland

The neoplastic cells of cutaneous T cell lymphomas (CTCL) express type 2 cytokines and are attacked by type 1 cytokine expressing reactive lymphocytes. Since imiquimod induces IFNα, IFNγ and IL12 and down-regulates IL4 and IL5, we asked whether it would be effective in CTCL treatment. Ten patients suffering from MF stage IA-IIA were treated by application of 5% imiquimod ointment (one lesion of 20 cm^2, 5 times per week for 12 weeks). Effects were assessed clinically and by evaluation of skin biopsies taken before and 4 weeks after treatment (treated and untreated lesion). Of the 7 patients completing the study, two developed a clinical response (1 CR, 1 PR). Two additional patients showed complete clearing of the treated lesion. Histological evaluation revealed an increase in CD8+ cells paired with a reduced number and a loss of epidermotropism of CD4+ cells in the responding lesions. Assessment of the lesional mRNA expression of IFNγ and IL4 by real-time PCR revealed a strong reduction of both, IFNγ and IL4 in treated and untreated lesion of the complete responder, thereby indicating complete clearing of the lesions. In treated lesions of local responders, an induction of IFNγ mRNA paired with only slight reduction of IL4 was observed. This may correlate with failure to induce a sufficient anti-tumour response despite induction of type 1 cytokines. The increase of IL4, found in treated and untreated lesions of non-responders, may indicate ongoing proliferation of type 2 tumour cells. In summary, our data prove principle and effectiveness of topical imiquimod therapy in CTCL. However, treatment should be extended to at least one lesion per skin region to increase the overall response rate.

P090

Proliferating antigen presenting cells expressing DC-SIGN (CD209+) in the decidua of early human pregnancy

U. Kaemmerer[1], A. O. Eggert[2], M. Kapp[1], A. D. McLellan[1], T. B. Geijtenbeek[3], J. Dietl[1], Y. van Kooyk[3], E. Kaempgen[2]

[1]Univ. Dep. Gynecology, 97080 Wuerzburg, Deutschland
[2]Univ. Dep. Dermatology, 97080 Wuerzburg, Deutschland
[3]Univ. Dep. of Molecular Cell Biology, 2100 Amsterdam, Netherlands

The maternal immune system tolerates the allogeneic embryo without general immunosuppression, thus intact human pregnancy can be regarded as a model for immunological tolerance. Since dendritic cell (DC) subsets could be involved in placental immune regulation, the uterine mucosa (decidua) was investigated for DC populations. Here we describe the detailed immunohistochemical and functional characterisation of HLA-DR positive antigen presenting cells (APC) in early pregnancy decidua. In contrast to classical macrophages and CD83+ DC, which were found in comparable numbers in decidua and non-pregnant endometrium, only decidua harboured a significant population of HLA-DR+/DC-SIGN+ APC further phenotyped as CD14+/CD4+/CD68+/-/CD83-/CD25-. These cells exhibited a remarkable proliferation rate (9.2% of all CD209+ cells) by double staining with Ki67. Unique within the DC-family, the majority of DC-SIGN+ decidual APC were observed in situ to have intimate contact with CD56+/CD16-/ICAM-3+ decidual NK-cells, another "pregnancy-restricted" cell population. In vitro, freshly isolated CD14+/DC-SIGN+ decidual cells efficiently took up antigen, but could not stimulate naive allogeneic T-cells at all. Treatment with an inflammatory cytokine cocktail resulted in downregulation of antigen uptake capacity and evolving capacity to effectively stimulate resting T cells. FACS analysis confirmed the maturation of CD14+/DC-SIGN+ decidual cells into CD25+/CD83+ mature DC. In summary, this is the first identification of a uterine immature DC-population expressing DC-SIGN, that appears only in pregnancy associated tissue, has a high proliferation rate and a conspicuous association with a NK-subset.

P091

Mast cells express a novel isoform of TLR2 and exhibit selectively impaired responses to TLR2/TLR6 dependent stimulation.

M. Metz[1], K. Grote[2], H. Peter[1], J. Knop[1], M. Maurer[1]

[1]Johannes Gutenberg University, Dept. of Dermatology, 55131 Mainz, Deutschland
[2]Medical School, Dept. of Cardiology and Angiology, 30625 Hannover, Deutschland

Mast cells (MCs) have been found to express toll like receptors (TLRs) 2, 3, 4, 6, 8, and 9, implicating an important role of MCs in innate immunity against pathogens. This view is supported by recent findings showing that MCs are activated by bacterial signals, i.e. *Staph. aureus* peptidoglycans, via TLR2. Here, we asked whether MCs protect from mycobacteria infections. Specifically, we tested whether MCs are activated by the *Mycoplasma* lipoprotein MALP-2, which requires TLR2/TLR6 signalling. Unexpectedly, murine peritoneal MCs failed to degranulate after stimulation with MALP-2, suggesting impaired functions of TLR2/TLR6 in MCs. When we performed RT-PCR analyses for TLRs and MyD88, MCs were found to express normal expression of 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. These observations are supported by our *in vivo* findings showing that MALP-2 induces equally strong inflammatory reactions after intradermal injections in normal or genetically MC-deficient $Kit^W/Kit^{W\text{-}v}$ mice ($Kit^W/Kit^{W\text{-}v}$, after 1h: 57.5 ± 12.8µm, vs. $Kit^{+/+}$: 64.1 ±11.4µm, p=0.72). This suggests that MCs unlike other skin cell populations can not respond to MALP-2/TLR2/TLR6 mediated stimulation (directly or indirectly) and, thus, do not contribute significantly to TLR2/TLR6 mediated inflammation by MALP-2. Our data indicates that MCs show impaired responses to TLR2/TLR6 dependent stimulation that are most likely explained by a MC specific new truncated isoform of TLR2. Further investigations are required to identify the unique structure of MC-TLR2 and its possible functions in MCs.

P092

Immunosuppression by induction of heme oxygenase 1: *In vitro* mechanism and effect in models of allergic contact dermatitis

J. Listopad[1], C. Schoenbein[1], R. Sabat[1], K. Asadullah[1], W. Doecke[1]

[1]Schering AG, Dept. of Exp. Dermatology, 13442 Berlin, Deutschland

The strong antiinflammatory and immunosuppressive potency of the stress protein heme oxygenase 1 (HO-1, HSP 32) has been proven in many models of inflammatory injury and autoimmunity. Hitherto, the underlying immune mechanisms have been poorly understood, and no data are available about the effect of HO-1 induction in models of inflammatory dermatoses.In our study, the potent HO-1 inducer Cobalt-Protoporphyrin IX (Co-PP) strongly suppressed T-cell proliferation in mixed lymphocyte reaction (MLR). This inhibition was due to a depression of antigen-presenting capacity, accessory molecule expression, and stimulatory cytokine secretion of monocytes. *In vivo*, dendritic cells (DC) represent the main antigen-presenting cells. Remarkably, the HO-1 inducer Co-PP led to an almost complete inhibition of the differentiation, maturation, and function of monocyte-derived dendritic cells. So, a strong decrease of the expression of DC markers (CD1a, CD83) and accessory molecules (HLA-DR, CD86) was observed accompanied with an increase of the expression of phagocytic receptors (CD14, CD16). Together these changes indicated a switch of the DCs to an immature and macrophagic phenotype. In fact, the antigen-presenting capacity of Co-PP-treated monocyte-derived DCs was strongly diminished in MLR with allogeneic CD4+ T-cells. The *in vivo* immunosuppressive activity of HO-1 induction was tested in DNFB (Type 1) and TMA (Type 2) model of ear inflammation in mice. Co-PP treatment at 24h before challenge dose-dependently depressed ear edema, infiltration, and cytokine levels. Importantly, Co-PP also strongly suppressed T-cell-dependent inflammation after challenge when applied around the sensitisation phase to prevent the initiation of the specific immune response. We hypothesise that the inhibition of DC differentiation, maturation, and function is the crucial mechanism for this immunosuppressive activity of HO-1 induction which may represent a novel approach for the treatment of T-cell mediated dermatoses like psoriasis, contact eczema, and atopic disease.

P093a

Expression Patterns of Tight Junction Proteins in Merkel Cell Carcinoma

N. K. Haass[1], P. Houdek[1], E. Wladykowski[1], I. Moll[1], J. M. Brandner[1]

[1]University Hospital Hamburg-Eppendorf, Department of Dermatology and Venerology, 20246 Hamburg, Deutschland

Despite the dogma, that the barrier function of the epidermis is held upright only by the stratum corneum, i.e. corneocytes and lipids, it recently has been shown, that cell-cell junctions, i.e. tight junctions and desmosomes, might very well be involved in this function too. In simple epithelia and endothelia tight junctions are responsible for the formation and maintenance of the tissue barrier between distinct compartments, e.g. the blood-brain-barrier. Tight Junctions are composed of various transmembrane proteins (Claudin 1-20, Occludin, junctional adhesion molecule (JAM)) and plaque proteins (Zonula occludens proteins 1-3 (ZO-1-3), Symplekin).
As previously shown by us and others in normal interfollicular epidermis Occludin and ZO-1 are expressed in the stratum granulosum and the transition layer, while Claudin 1 occurs in all layers. There is evidence suggesting that tight junctions play a role in tumour biology. Recently we showed changes in expression patterns of tight junction proteins in malignant melanoma and adjacent epidermis compared to melanocytic nevi. There is not much known about the expression patterns of tight junction proteins in Merkel cell carcinoma.
So in this work we focus on the occurrence of Claudin 1, 3, 4, and 5, Occludin and ZO-1 in Merkel cell carcinoma.
Investigations were performed by immunofluorescence microscopy of frozen sections and of cultured cells using previously described specific antibodies.
Claudin 3, 4, and 5, Occludin and ZO-1 were found in Merkel cell carcinoma cells. The expression of these molecules might result in functional tight junctions and therefore play a role in the isolation of the tumour from its environment.
Claudin 1 is not found in Merkel cell carcinoma cells. This is a surprising result, because Claudin 1 is the only tight junction protein that appears in the environment of Merkel cells: the basal layer of the epidermis.
The endothelial marker Claudin 5 is found in vessels but also in Merkel cell carcinoma cells in certain parts of Merkel cell carcinoma. This result reminds on the "vessel formation" by other malignant tumours such as malignant melanoma. So this might be a hint, that there occurs such a "vessel formation" in Merkel cell carcinoma and that Claudin 5 is involved this process.

P093b

Expression Patterns of Connexins in Merkel Cell Carcinoma and Adjacent Epidermis

N. K. Haass[1], P. Houdek[1], J. M. Brandner[1], I. Moll[1]

[1]University Hospital Hamburg-Eppendorf, Department of Dermatology and Venerology, 20246 Hamburg, Deutschland

Gap junctions (GJ) play an important role in cell communication. They form channels between adjacent cells allowing the diffusion of small molecules including ions, metabolites and second messengers. GJ consist of two hemichannels called connexons (one of each cell), each formed by six polypeptides called connexins. The type of connexins forming GJ influences the selectivity of the channels and thereby controls the specificity of cell-cell-communication. Ten of the known 21 connexins are expressed in human skin. GJ have been supposed to be involved in the regulation of keratinocyte growth, differentiation and migration. There is evidence suggesting that connexins play a role in tumour biology.Because of the shown changes of the expression pattern of Cx26 and Cx30, e.g. in hyperproliferative epidermis, we were interested in the distribution of Cx26 and Cx30 in skin tumours and their adjacent epidermis. Because of the ubiquitous expression of Cx43 in normal skin we also were interested in its expression in skin tumours. Recently we showed a change in the expression pattern of connexins in Basal and Squamous Cell Carcinoma and Malignant Melanoma. There is not much known about connexins in Merkel Cell Carcinoma (MCC) and adjacent epidermis. Investigations were performed by immunofluorescence microscopy using previously described specific antibodies.As previously shown Cx30 and Cx26 are only weakly, if at all, expressed in normal interfollicular epidermis, while Cx43 is expressed ubiquitously in all cell layers.Now we show that neither Cx30 and Cx26 nor Cx43 do occur in MCC. Cx43 is expressed troughout the interfollicular epidermis adjacent to and as well distant from the MCC. There is no expression of Cx30 and Cx26 in interfollicular epidermis distant from the MCC. In contrast, Cx30 and Cx26 are expressed in the interfollicular epidermis adjacent to MCC. These results show an induction of Cx30 and Cx26 in interfollicular epidermis adjacent to MCC. Previously we demonstrated similar results investigating GJ proteins in the epidermis adjacent to other malignant skin tumours, but not to semimalignant and benign lesions. So one might suggest that the change of the composition of GJ in the epidermis adjacent to malignant skin tumours may play a role in the metastasation of these tumours.

P095

***Pseudomonas aeruginosa* selectively induces a novel interferon α/β-inducible responsive gene in human keratinocytes**

B. F. Goethel[1], L. Schwichtenberg[1], J. M. Schroeder[1], J. Bartels[1]

[1]Hautklinik Kiel, 24105 Kiel, Deutschland

Skin keratinocytes are the first line of defense against invading microorganisms. In order to investigate keratinocyte mechanisms to differentially respond to and defend against microorganisms we looked for keratinocyte genes differentially expressed upon contact with secreted pathogen-associated molecules (PAMs). Normal human keratinocytes (NHK) were stimulated with supernatants of cultured *Pseudomonas aeruginosa* (*P.a.*), *Staphylococcus aureus* (*St.a.*) and *Candida albicans* (*C.a.*). NHK gene expression was assayed using an improved systematic differential display PCR method (SDD). Silver-staining of the electrophoretically separated SDD-PCR-product pattern was performed to identify differentially expressed keratinocyte genes. Verification of differentially expressed genes was accomplished by using gene-specific semi-quantitative RT-PCR. As one result, we found transcriptionally upregulation of IFI60/ISG60/IFIT4, a new IFN-response gene, following keratinocyte stimulation with *P.a.*-supernatant, but not with *St.a.*- and *C.a.*-supernatant.We found IFI60 upregulation depending on conditions of bacterial growth (including static or suspension culture) and the particular *P.a.*-strain used. Strongest IFI60-upregulation was seen when *P.a.* was grown in a Mg^{2+}-low medium under static culture condition. Keratinocyte stimulation with TNF α, Interleukin 1β (IL-1β), phorbol 12-myristate 13- acetate (PMA) and IL 6 resulted in no induction of IFI60, indicating that gene regulation of IFI60 differs from that of human beta-defensin 1 (hBD1), hBD2 and hBD3 which are known for their antimicrobial activities. Retinoic acid, known for modulating expression of genes involved in cell differentiation, also was not found to modulate IFI60-expression. Lipopolysaccharide (LPS) did not upregulate IFI60-expression, indicating that LPS is not the IFI60-inducing factor. Stimulation with interferons α,β or γ, which are known for their role in host defense, resulted in upregulation of IFI60 in NHK`s and (except for IFNγ) in HaCaT. Future investigations (using HPLC purified fractions of active *P.a.*-supernatants) will include the characterisation and identification of the molecular nature of the *P.a.*derived stimulus to help explain the differential regulation and function of the IFI60 gene in keratinocytes upon contact with *P.a.*.

P096

Large scale generation of mature monocyte-derived dendritic cells for clinical application in cell factories

T. G. Berger[1], E. Strasser[2], B. Feuerstein[1], D. Schreiner[1], G. Schuler[1], B. Schuler-Thurner[1]

[1]Universitätsklinik Erlangen, Dermatologische Klinik mit Poliklinik, 91052 Erlangen, Deutschland
[2]Universitätsklinik Erlangen, Abteilung für Transfusionsmedizin, 91054 Erlangen, Deutschland

Dendritic cells (DC) are increasingly used for the immunotherapy of cancer. Both the induction of tumor-specific T cells and some clinical regressions have been observed in early phase I/II trials by using either DC isolated from blood, DC generated from CD34+ precursors ex vivo, and 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 overcome prior limitations such as the repetitive, labor-intensive generation of DC in a large number of open culture vessels. We describe here as a result of several years of optimization in detail a procedure that uses the so-called Nunc cell factories to process a whole apheresis product labor- and cost-effectively in a quasi closed system to reproducibly generate (by using GM-CSF + IL-4 followed by a maturation cocktail composed of IL-1b + IL-6 + TNF-α + PGE2) large numbers (8,32 +/- 3,8 % of input PBMC) of mature (> 85% CD83+), monocyte-derived DC that can be successfully cryopreserved. Our report is based on the processing of >100 aphereses including 52 unselected aphereses in advanced melanoma patients. This allows us also to suggest meaningful quality and validation criteria. The DC generation method appears particularly promising as respective DC-vaccination proved already immunogenic in cancer patients, and the cell factories can readily be converted to a fully closed system by using appropriate valves, tubings and bags.

P097

Bimosiamose, a pan-selectin antagonist improves disease manifestation both in human psoriatic skin SCID mice and psoriatic patients - selectin antagonists as a new treatment strategy for inflammatory diseases

M. Friedrich[1], G. Wolf[2], S. Philipp[1], N. Schoetzau[1], K. Berens[3], S. Kang[4], T. N. Dam[4], R. Zahlten[2], W. Sterry[1]

[1]University Hospital Charité, Dept. of Dermatology and Allergy, 10117 Berlin, Deutschland
[2]Revotar Biopharmaceuticals AG, 16761 Hennigsdorf, Deutschland
[3]Texas Biotechnology Corporation, 77030 Houston, Tex., USA
[4]The University of Michigan, Dept. of Dermatology, 48109 Ann Arbor, USA

The selectin family of vascular cell adhesion molecules is comprised of structurally related carbohydrate binding proteins, which mediate the initial rolling or "tethering" of leukocytes on the vascular endothelium following inflammation or injury. In T-cell-mediated diseases this process is one of the crucial events in initiating and maintaining inflammation. Therefore, selectins are an attractive target for the development of new anti-inflammatory therapeutics. We investigated bimosiamose, a synthetic pan-selectin antagonist in psoriasis. Using the human psoriatic skin-SCID mouse transplant model, bimosiamose caused a decrease of disease severity as well as prevention of psoriasis in symptomless psoriatic skin. Therefore, in a phase-II open clinical trial, we administered bimosiamose (600mg/day) over 14 days in five psoriatic patients. As demonstrated by assessment of psoriasis area and severity index (PASI), patients demonstrated significant improvement at the end of the study ($p=0.02$) which was paralleled by a reduction of epidermal thickness, a decrease of $CD3^+$ and $CD4^+$ cells and a reduction of P-selectin expression in immunohistochemical analysis. Flow cytometric analyses showed a reduction of peripheral CD45RO+ T-cells. Assessment of safety parameters showed no abnormal findings during the time of therapy. These data suggest that selectin antagonists may be a promising strategy for the treatment of inflammatory diseases.

P099

Induction of vitiligo in patients with advanced malignant melanoma after intranodal injection of autologous tumor lysate-pulsed dendritic cells is associated with generation of melanocyte antigen specific effector memory cells

T. Maier[1], A. Tun-Kyi[1], M. Gilliet[1], R. Dummer[1], G. Burg[1], F. Nestle[1]

[1]Unispital Zürich, Dermatologie, 8091 Zürich, Schweiz

Vaccination with peptide pulsed autologous dendritic cells (DC) has generated T cell responses and in selected cases clinical responses in patients with advanced metastatic melanoma. The use of tumor lysate as tumor antigen has the potential advantage of a broader antigen repertoire and is independent of the HLA-type of a given patient. We treated 17 stage IV melanoma patients with 6 intranodal injection of autologous tumor lysate-pulsed mature dendritic cells. Objective clinical responses were observed in four patients (24%), with one additional patient experiencing a mixed response. Eight out of 17 (47 %) patients developed characteristic vitiligo lesions in acral and periorificial localization. Lesions progressed after stopping therapy. Biopsies of vitiligo lesions showed absence of melanin with CD8+/TIA-1+Granzyme B positive lymphocytic infiltrates at the dermo-epidermal junction. There was no clear correlation of vitiligo development and clinical response. Induction of DTH reactivity to autologous tumor lysate was observed after vaccination. Tetramer analyis in HLA-A2 patients showed expansion of MelanA positive as well as gp 100 effector CD8 T cells only in patients developing vitiligo. Furthermore, maturation of T cells with acquisition of an effector memory phenotype (MelanATetramer+/CCR7-/CD45RA-) after DC therapy was demonstrated.
We show that occurrence of vitiligo in a high percentage of patients vaccinated with tumor lysate pulsed DC is associated with the expansion of melanocyte antigen specific effector memory cells.

P100

Tryptophan-dependent synthesis of isomeric bisindolylderivatives by *M. furfur*

P. Mayser[1], H. Stapelkamp[1], W. Thoma[1], H. Krämer[1], B. Irlinger[2], W. Steglich[2]

[1]Zentrum für Dermatologie und Andrologie und Klinische Pharmakologie, Justus-Liebig-Universität Giessen, Poliklinik, 35385 Giessen, Deutschland
[2]Institut für Organische Chemie der LMU München, 81377 München, Deutschland

As the main nitrogen source in *Malassezia furfur*, tryptophan induces the formation of fluorochromes and pigments. The Trp-dependent pigment formation might explain some hitherto unelucidated phenomena of pityriasis versciolor.
For the investigation of the fluorochromes, *M. furfur* (CBS 1878) was incubated at 30° for 14 days on a pigment-inducing medium. The agar extract was purified by column chromatography, preparative TLC and HPLC. The structures of the pure metabolites were determined by mass spectrometry and NMR spectroscopy. A pale yellow compound eluting from the column with 22% acetonitrile was found to exhibit a strong green-yellow fluorescence. The fluorochrome is a new bisindolyl compound. It was named pityrialacton ($C_{20}H_{12}N_2O_3$) because of its furan-2,3-dione structure. As shown by the fluorescence spectrum with excitation maxima at 366 nm, pityrialactone appears to be responsible for the green-yellow fluorescence of pityriasis versicolor lesions under Wood light. Additionally, the mesomeric system of pityrialactone could serve as a free radical trapper. Pityrialactone is accompanied by the isomeric bisindolylmaleic anhydrid (pityriaanhydride), which has not yet been described as a natural product but is a known intermediate in the total synthesis of bisindolylmaleimides.

P101

Protein profiling of therpeutic effects in the serum of melanoma patients treated by angiostatic chemotherapy (daily trofosfamid, rofecoxib, pioglitazone) by SELDI ProteinChip® technology

B. Becker[1], R. Bogumil[2], M. Landthaler[1], W. Stolz[1], A. Reichle[3], T. Vogt[1]

[1]Universitaet Regensburg, Dermatologie, 93042 Regensburg, Deutschland
[2]Ciphergen, GU2 7YG Guildford, England
[3]Universitaet Regensburg, Onkologie, 93042 Regensburg, Deutschland

Genome and transcriptome analyses have revealed that the initiation and progression of malignant melanomas depend on a huge variety of molecular deregulations, which in part can impact the composition of serum proteins in advanced tumor stages. In a therapeutic setting early surrogate markers of a possible response to a certain regimen would be a tremendous progress that would revolutionize the clinical management of stage IV MM patients in dermato-oncology. The newly developed "surface enhanced laser desorbtion / ionisation" (SELDI) technology coupled to a chip-based retention-chromatography of proteins provides a feasable monitoring of biochemical features simultaneously with the size of proteins by "time-of-flight"-mass spectrometry (TOF-MS).
To identify potential surrogate markers for the characterization of the effects of angiostatic palliative chemotherapy in patients with metastasising melanomas the protein profiles in sera of 6 patients at the begin and during the course of this treatment were analysed. After normalization of the protein profiles 7 differentially expressed proteins with sizes ranging from 4.8 kDa to 70 kDa were detected. One peptide of 7.8 kDa was downregulated during therapy in 5 out of 6 cases. Peptides of 4.8 kDa, 5 kDa, 7.8 kDa and a protein of 70 kDa were downregulated in 50 % of all cases. Two peptides of 5.9 kDa and 8.6 kDa were induced during therapy.
We conclude, the SELDI ProteinChip technology enables a quick analysis of protein profiles in sera, which can describe the complex changes during tumor progression and the therapeutic intervention. Larger correlational studies are needed to identify the desired surrogate markers that may reflect a favourable response after initiation of therapy.

P102

Imiquimod 5% anal tampons - an alternative option to surgery for intraanal condylomata

A. Salat[1], H. Pokorny[1], R. Kirnbauer[2], F. Herbst[1]

[1]University of Vienna Medical School, Department of General Surgery, A-1090 Vienna, Oesterreich
[2]University of Vienna Medical School, Immunodermatology, Laboratory of Viral Oncology, A-1090 Vienna, Oesterreich

Destructive therapies in the anal canal are afflicted by a high morbidity including postoperative pain, stenosis and loss of sensation. Immunomodulation by imiquimod has become an accepted therapy concept for the treatment of condylomata acuminata and its use as anal tampons as an adjunct to surgery has been described.
We investigated the clinical and virologic effect of imiquimod 5% incorporated into anal tampons (overnight application for 3 month) in 86 patients (n=74 men (86%) and n=12 women (14%)) for condylomata acuminata of the anal canal. Twentythree patients were HIV-positive (28%), gay or bisexual orientation was reported by 55 (77% of males).
Local irritation (perianal rubor, itching) was a common side effect reported by 47 patients (55%). Systemic symptoms (tiredness, arthritis, muscle pain) prompted a temporal break in therapy in 11 patients (13%), 4 patients of those (5%) stopped treatment and were excluded from further analysis. Overall, pretreatment intraanal swabs were positive for human papillomavirus (HPV) types in all patients by hybrid capture II test. High-risk-HPV types only were detected in 5 patients (6%), low-risk-HPV only in 37 patients (46%), and 39 patients (48%) were positive for both types. Squamous intraepithelial lesions were detected in 13 patients (16%) in biopsies from the dentate line (AIN-I: 9%; AIN-II 3.5%; AIN-III: 3.5%).
After 3 months of therapy all warts were clinically cleared in 49% of patients (n=40), 24 (30%) of them had also become negative for HPV DNA. Clinical improvement reflected by reduction of wart number and/or size occurred in 22 patients (27%), stable disease in 11 (14%) and warts had progressed in 8 (10%) patients.
This is the first report on imiquimod 5% anal tampons as mono-therapy for condylomata acuminata of the anal canal. Although flu-like symptoms possibly related to therapy occured in a minority of patients, the lower rate of local side effects, compared to topical imiquimod 5% cream, may improve compliance. Although this study is limited by short observation and follow-up periods, our results indicate that imiquimod 5% anal tampons offer a safe and well-tolerated alternative option to destructive therapies for HPV induced lesions of the anal canal.

P103

The association of psoriasis vulgaris with internal diseases

D. Schmitt[1], K. Carstensen[2], R. Bugdahl[1], E. Christophers[1], M. Weichenthal[1]

[1]University of Kiel, Dpt. of Dermatology, 24105 Kiel, Deutschland
[2]University of Kiel, Dpt. of Medical Statistics, 24105 Kiel, Deutschland

Several associations of psoriasis with different types of other diseases have been described. These data may be helpful in the clinical management of patients and allow important insights into common pathophysiological pathways, but the evidence so far is conflicting. The higher prevalence of inflammatory bowel disease in patients with psoriasis is well established, but occurs only in a minority of cases. Of more clinical importance, but still not clearly defined are associatins with other, more common disorders, e.g. diabetes mellitus and cardiovascular disease.
In a hospital-based study 626 patients with psoriasis vulgaris were evaluated for the prevalence of metabolic, cardiovascular and autoimmune disease. These data were compared to a set hospital controls, attending the clinic for surgery of primary skin tumours. For further validation, all factors found to be correlated in the primary analysis were included in a multivariate comparison with a population-based sample of 7,124 individuals.
In the primary analysis, patients with psoriasis showed a significantly enhanced odds ratio (OR) for the prevalence of diabetes mellitus, arterial hypertension, and hyperlipedemia as compared to the hospital-based controls. Smoking and the regular consumption of alcoholics were also significantly more prevalent in psoriasis patients. In contrast, the body mass index (BMI) and the occurence of less common disorders were not significantly different between the groups. After controlling for the presence of these factors in a multivariate analysis and comparison to the population-based sample, diabetes mellitus, smoking and alcohol consumption remained significantly associated with psoriasis. The incresed OR for diabetes was similar in males and females, but not not homogeneously distributed over all age classes. Specifically, the prevalence of diabetes in younger ages was strongly elevated.
In summary, our findings confirm earlier reports of psoriasis being associated with diabetes mellitus, smoking and alcohol consumption. The association of psoriasis with diabetes may be confined to the genetically determined psoriasis type 1.

P104

Hybrid cell vaccination in metastatic melanoma: Clinical and immunologic results of a phase I/II study

H. A. Haenssle[1], S. Emmert[1], M. Zutt[1], L. Kretschmer[1], H. Schmidberger[2], S. W. Krause[3], R. Andreesen[3], A. Soruri[4]

[1]Georg-August University Goettingen, Dermatology, D-37075 Goettingen
[2]Georg-August University Goettingen, Radiotherapy, D-37075 Goettingen
[3]University of Regensburg, Hematology and Oncology,D- 93042 Regensburg
[4]Georg-August University Goettingen, Immunology, D-37075 Goettingen

Hybrid cell vaccination with fusion products of autologous tumor cells and mature allogeneic MHC II bearing dendritic cells (DCs) has been described to induce cytotoxic T lymphocyte (CTL) mediated immune responses. Such hybrid cells provide both - the full antigenicity of the tumor cells and the strong immunogenicity of allogeneic MHC molecules on DCs. Allogeneic MHC molecules should activate and expand alloreactive T-helper cells required for the induction of long lasting CTL mediated immunity. The aim of this study was to assess safety, anti-tumor activity and immune responses of a hybrid cell vaccine in patients with disseminated malignant melanoma.
PATIENTS / METHODS: In a phase I/II study, we treated 11 patients by monthly intracutaneous or subcutaneous application of a hybrid cell vaccine generated by electrofusion of autologous melanoma cells with mature allogeneic dendritic cells. In addition patients received subcutaneous low dose interleukin-2 (IL-2) injections for 6 days following each vaccination.
RESULTS: No serious side effects were observed. Ten patients showed progressive disease and one patient experienced a short lasting stable disease. None of the patients developed a positive delayed type hypersensitivity reaction against irradiated autologous melanoma cells. Two patients were monitored in more detail using 3[H]tymidine proliferation assay, cytokine secretion assay and cytotoxicity assay without evidence for induction of a specific anti-melanoma T cell response.
CONCLUSION: No unequivocal beneficial effects of the utilized hybrid cell vaccine could be demonstrated.

P105

Effects of a Chemical Sunscreen on UV-Induced Changes of Dermoscopic Features in Melanocytic Nevi

I. Zalaudek[1], S. Grinschgl[1], P. H. Soyer[1], J. Smolle[1], H. Kerl[1], P. Wolf[1], R. Hofmann-Wellenhof[1]

[1]Universitätsklinik für Dermatologie und Venerologie, Graz, A-8036 Graz

Exposure to UV irradiation can lead to different changes in melanocytic nevi. In this study we investigated the effect of a sunscreen on the UV irradiation-induced changes of dermoscopic features in melanocytic nevi.

Twenty two melanocytic nevi were exposed to three minimal erythema doses (MEDs) of solar simulated UV irradiation. One half of each nevus was treated with a sunscreen. The other half was unprotected during UV irradiation. Digital dermoscopic images were taken before and 3 and 7 days after UV irradiation. The dermoscopic images were evaluated for the presence of dermoscopic changes by two experienced dermoscopists. The review of the images were done without access to any information except the image on the screen.

Three days after UV irradiation, both halves developed an erythema, but it was less severe in the sunscreen treated half. Additionally, a fading of the pigment network was seen in both halves, however, it was also less manifest in the sunscreen protected sites.
Seven days after UV irradiation a number of complex dermoscopic changes were observed. In particular, the UV irradiated melanocyctic nevi showed an increasing number of irregularly distributed brown to black globules, grey to brown to black dots, grey blue areas, as well as an irregular atypical pigment network with broadened network and new occurrence of vessels. These changes were also observed in the sunscreen protected halves, but the changes were significantly less evident.

In conclusion, UV irradiation of melanocytic nevi with three MEDs induces transient changes in their dermoscopic appearance that are sometimes suggestive of malignant melanoma. These changes can be prevented in part by a chemical sunscreen.

P106

Increased keratinocyte expression and serum levels of calcium-binding proteins S100A8 and S100A9 in psoriasis

S. Benoit[1], A. Toksoy[1], M. Schmidt[2,1], J. Roth[3], E. Bröcker[1], S. Ludwig[2], R. Gillitzer[1], M. Goebeler[1]

[1]University of Würzburg, Department of Dermatology, D-97080 Würzburg
[2]University of Würzburg, Institut für Medizinische Strahlenkunde und Zellforschung (MSZ), 97078 Würzburg, Deutschland
[3]University of Münster, Institute of Experimental Dermatology, D-48149 Münster

S100A8 and S100A9, two calcium-binding proteins of the S100 family, have originally been described in cells of myelomonocytic origin where they are expressed in a differentiation-dependent manner. Their genomic localization in the epidermal differentiation complex at the 1q21 locus prompted us to study S100A8/S100A9 expression in a skin disease with abnormal keratinocyte differentiation and hyperproliferation, i. e. psoriasis. Using immunohistochemistry and in situ-hybridization, normal interfollicular epidermis was found to express only marginal amounts of S100A8 and S100A9 mRNA and protein. In psoriatic skin, however, a dramatic induction of both mRNA and protein expression could be detected in suprabasal layers of the epidermis. Since S100A8/S100A9 are known to be secreted by keratinocytes we wondered whether their strong up-regulation in psoriatic skin is reflected by elevated serum levels in these patients. Employing ELISA, serum levels of S100A8/S100A9 heterodimers were determined in normal control subjects and in psoriasis patients and found to be significantly elevated in the latter. Serum levels appeared to correlate with the severity of the disease as evaluated by the psoriasis area and severity index (PASI).Our results indicate that abnormal hyperproliferation of keratinocytes in psoriatic skin is associated with a strong up-regulation of the calcium-binding proteins S100A8 and S1009 in lesional keratinocytes which is reflected by elevated serum levels of both proteins.

P107

Uptake of 3H-imipramine versus 3H-haloperidol in pigment-producing and non-pigment-producing melanoma cell-cultures and investigations on the drug association with the cellular constituents

D. Magnani[1], I. Moll[2], G. Skopp[3], L. Pötsch[1]

[1]Institute of Legal Medicine, Johannes-Gutenberg University, 55131 Mainz, Deutschland
[2]Department of Dermatology and Venerology, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Deutschland
[3]Institute of Legal Medicine, Ruprecht-Karls University, 69115 Heidelberg, Deutschland

In recent years hair fibers have been shown to entrap and to store organic substances present in the organism during hair growth. The detailled mechanisms of drug uptake into the various cell types present in the hair follicle need to be clarified. The aim of the present study was to compare the drug uptake and drug association with particular cell constituents of two model substances i.e. imipramine (high lipophilicity) and haloperidol (high melanin affinity) in pigment and non-pigment producing cells (melanotic - amelanotic B16/ F1 cell culture). Moreover the drug incorporation into melanin was investigated by extraction experiments. Drug uptake was initially dependent on the physico-chemical parameters of both model substances, on their concentration in the cell culture media as well as on cell pigmentation. The drug uptake of imipramine was always higher than that of haloperidol. Pigment-producing cells showed no tendency to saturation. In non-pigment producing cells no differences were observed for association with the cell constituents between the drugs tested. For pigment-producing cells different pattern were found for the two substances. These findings underlined that pigments are carriers for drug molecules and therefore melanins influence the drug incorporation in growing hair. In conclusion, cell culture experiments seem a suitable approach to understand drug uptake and the influence of pigmentation on hair analysis.

P108

Upregulation of CYP1B1, CYP3A, TAP1 and TAP2 in cultured lymphocytes from patients with porphyria cutanea tarda after in vitro-stimulation with interferon alpha

J. Frank[1,2], P. Poblete[1,2], M. M. Neis[1], A. Rübben[1], H. F. Merk[1,2], J. M. Baron[1]

[1]University Clinic of the RWTH, Dept. of Dermatology, D-52074 Aachen
[2]University Clinic of the RWTH, Interdisciplinary Center for Clinical Research (IZKF), 52074 Aachen, Deutschland

Porphyria cutanea tarda (PCT) is the most frequent disorder of porphyrin-heme biosynthesis worldwide. Two forms of PCT are currently distinguished: Sporadic/acquired (type I) PCT and familial/inherited (type II) PCT. Both types of PCT arise from a decreased activity of the enzyme uroporphyrinogen decarboxylase (URO-D), leading to photosensitization on the sun exposed areas of the skin. However, the enzymatic deficiency by itself is usually not sufficient for the development of cutaneous symptoms. Clinically overt disease rather requires the involvement of specific triggering factors. These factors are mainly alcohol, estrogens, iron and polychlorinated hydrocarbons. Interestingly, in a 63-year-old male with malignant melanoma we observed the occurence of PCT after an immunotherapy with interferon alpha (IFN-alpha). Mutation analysis in the URO-D gene was indicative of type II PCT. Subsequently, we cultured lymphocytes obtained from peripheral blood of this patient and also from a second patient with clinically overt type I PCT. Lymphocytes from both patients were stimulated in vitro at different concentrations with INF-alpha and phenobarbital, a further drug known to provoke PCT. Interestingly, we observed a significant upregulation of cytochrome P450 (CYP) 1B1 and CYP3A in both individuals. Further, an upregulation of CYP 3A5 was detected in the patient with type II PCT after stimulation with phenobarbital. mRNA-expression of the transport-associated proteins (TAP) 1 and 2 was upregulated in the individual with type II PCT after stimulation with IFN-alpha proving the modulating effect of long term immunotherapy on antigen presentation. However, this effect was not observed in the patient with type I PCT who did not receive INF-alpha. Although previous data obtained in hepatocytes indicated a downregulatory effect of INF-alpha on CYP expression, our results show that it rather leads to an upregulation of CYP expression in other tissues. Our data suggest that cytokines like INF-alpha, commonly used in low and high dose immunotherapy, represent as yet unrecognized mediators with the potency of triggering PCT.

P109

Gene transfer by a novel replication-defective and dominant-negative recombinant HSV-1 viral vector.

D. Hoeller[1,2], F. Yao[1]

[1]Harvard Medical School, Division of Plastic Surgery, 02115 Boston, MA, USA
[2]Universitaetsklinik der RWTH Aachen, Hautklinik, 52074 Aachen, Deutschland

Herpes simplex virus (HSV)-1 replicates in epithelial cells and establishes life-long latent infection in neuronal cell bodies within the sensory ganglia of infected individuals. In light of its dual-life cycle and its ability to infect a broad range of mitotic and post mitotic cells, HSV-1 has recently gained attention as a potential genetic vehicle to deliver and express therapeutic genes in a variety of tissues. Because of its large genome size, up to 30-50 kb transgene can be packed into recombinant HSV-1 vectors. At present, two major classes of HSV-1 vectors have been developed: 1) replication-defective viruses, and 2) replication conditional mutants. The ability of HSV and these mutant recombinants to establish life-long latent infections and the fact that they are replication-competent in the context of wild-type HSV-1, do, however, raise concerns in contemplating the use of these vectors in humans. A recombinant herpes simplex virus capable of inhibiting its own replication as well as replication of wild-type HSV would greatly increase its safety as a general-purpose vector for in vivo gene transfer, anti-tumor therapy, and a viral vaccine against HSV infection.
We generated a novel HSV-1 recombinant, CJ83193, by combining the gene encoding the dominant-negative mutant form of HSV-1 DNA replication origin binding protein UL9-C535C with the tetR-mediated transcription repression switch. CJ83193 expresses UL9-C535C under the control of the tetO-bearing hCMV major immediate-early promoter (Yao and Eriksson, 1999). The de novo synthesis of CJ83193 can be effectively suppressed by UL9-C535C peptides in non-tetR expressing cells, and is subject to tetracycline regulation in a range of four to five orders of magnitude in tetR-expressing cells.
By replacing the gene encoding UL9 with the LacZ gene, we generated an HSV-1 viral vector, CJ9-LacZ. We are presenting the infection efficiency and cell cytotoxicity of CJ9-LacZ at different multiplicity of infection (MOI) in the various cell types and demonstrate that CJ9-LacZ is a possible and efficient gene transfer vehicle to various cell types, like osteosarcoma cells, monkey kidney cells, human and porcine fibroblasts, as well as porcine keratinocytes. Furthermore we present the gene delivery and expression of CJ9-LacZ infected Keratinocytes into porcine full-thickness wounds.

P110

Changes in basement membrane components in hair follicles of pili annulati patients

K. A. Giehl[1], D. Ferguson[2], D. Dean[3], Y. H. Chuang[3], R. D. Dawber[3], I. Leigh[4], F. Wojnarowska[3]
[1]Churchill Hospital, Department of Dermatology, Headington, Oxford OX3 7LJ, UK
[2]Ludwig-Maximilians-Universität, Department of Dermatology, 80337 Munich, Deutschland
[3]Oxford University, Nuffield Department of Pathology, John Radcliff Hospital, Headington, Oxford OX3 9DU, UK
[4]St. Bartholomew´s & the Royal London School of Medicine and Dentistry, Centre for Cutaneous Research, London E1 2AD, UK

Pili annulati is a rare autosomal inherited hair shaft abnormality in which clinical examination reveals alternating light and dark bands leading to a shiny appearance of the hair. The light bands are the abnormal areas due to cavities within the cortex that appear dark when examined by transmitted light microscopy. The pathogenesis remains unclear.
Four cryopreserved scalp specimens of two affected individuals and six unaffected individuals undergoing cosmetic surgery were analysed for different keratins (6,10,14,18,19) and basement membrane components as collagen type VII, Laminin 5, Collagen IV Laminin 1, Fibrillin and Integrin α6β4 by immunofluorescent staining.
Staining for keratins 6, 10, 14, 18 and 19 showed no difference in staining intensity and quality of staining pattern.
While laminin 5 and collagen type VII appeared as a smooth band in the basement membrane zone in control sections, an undulating, wavy pattern was seen in the sections of individuals with pili annulati. These differences were also found in the sebaceous glands. Further analysis by transmission electron microscopy (TEM) showed a single thin electron dense band in the control sections and a ramification of reduplicated lamina densa situated between an epithelial cell with collagen fibres in the affected individuals.
In contrast to the original supposed keratin defects in the pili annulati hair shaft abnormality, our results strongly suggest structural difference in the basement membrane zone of affected individuals.

P111

Mu-Opioid Receptor 1A (MOR 1A) is present on cutaneous sensory nerve fibers of humans`

S. Ständer[1,2], M. Gunzer[1], D. Metze[1,2], T. Luger[1,2], M. Steinhoff[1,2]

[1]Ludwig-Boltzmann-Institut, 48149 Münster, Deutschland
[2]Universitäts-Hautklinik, 48149 Münster, Deutschland

Opioid peptides are endogenous neuromodulators which play a major role in the nociceptive pathway by interacting with opioid receptors. Four opioid receptors (mu-, delta-, kappa-, orphan-receptor) have been cloned with a wide distribution in the central and peripheral nervous system. In the present study, the presence of the mu-opioid receptor (MOR) isoform 1A could be shown in nerve fibers of human skin. Immunohistochemical analysis revealed MOR-immunoreactivity to be present in dermal and epidermal nerve fibers. Double immunofluorescence staining revealed that MOR is present on calcitonin gene-related protein-positive sensory nerve fibers while autonomic nerves of blood vessels, hair follicles or skin glands were negative. In diseased skin such as psoriasis vulgaris, atopic dermatitis, and prurigo nodularis, distribution of MOR 1A immunoreactivity was similar to that of normal skin. These findings expand our knowledge about a direct regulatory role of cutaneous opioid receptors in the skin. Thus, peripheral cutaneous opioid receptors may be involved in the transmission of pain and pruritus, respectively. This is supported by previous observation that opioid receptor antagonists may significantly diminish experimentally-evoked histamine-induced itch of the skin. Together, our findings contribute to the understanding of the high antipruritic potency of opioid receptor antagonists in various skin and systemic diseases.

P112

Modulation of STAT6 in human monocyte-derived dendritic cells by 1,25- dihydroxycholecalcifereol (vitamin D3).

K. Zhu[1], R. Gläser[1], U. Mrowietz[1]

[1]Universitätsklinikum Kiel, Abt. Dermatologie, 24105 Kiel, Deutschland

The active metabolite of vitamin D3, 1,25(OH)2D3, is a potent inhibitor of IL-4/GM-CSF-induced differentiation of human monocytes into dendritic cells. Antigen-presentation and lymphocyte proliferation in the mixed lymphocyte reaction is blocked by 1,25(OH)2D3-treatment.
Interleukin 4 is crucial inducing monocyte differentiation into a dendritic phenotype and to prevent macrophage-like development. Signal transduction of IL-4 at least in part is mediated through STAT6-protein. In this regard phosphorylation of STAT6 is important to activate this pathway.
Therefore we have investigated whether treatment of monocytes with 1,25(OH)2D3 leads to a modulation of STAT6 and phospho-STAT6 expression.
Elutriator-purified human monocytes were incubated with IL-4/GM-CSF to induce dendritic cell differentiation for 5 days. 1,25(OH)2D3 was added in concentrations from 10-8 to 10-13 mol/l from the beginning. Treatment with the solvent ethanol (0,1%, v/v), or medium alone served as controls. After incubation cells were harvested and subjected to protein extraction followed by western-blot analysis.
The results of our study clearly showed a significant and dose-dependent down-modulation of phospho-STAT6 protein-expression, not, however, of non-phosphorylated STAT6. The half-maximal inhibitory concentration (IC50) of 1,25(OH)2D3 was calculated to be 10-10 mol/l.
Our data provide evidence that the inhibition of dendritic cell differentiation by 1,25(OH)2D3 at least in part is mediated by inhibiting IL-4 signalling through a down-modulation of phospho-STAT6.

P113

Differential expression of TGFβs, TGFβ-receptors and SMAD-proteins in psoriasis skin

H. Yu[1], O. Bock[1], R. Gläser[1], U. Mrowietz[1]

[1]Universitätsklinikum Kiel, Abt. Dermatologie, 24105 Kiel, Deutschland

Transforming growth factor β (TGFβ) is an important growth factor which acts as an inhibitor of keratinocyte proliferation. On the other hand TGFβ stimulates fibroblasts to produce extracellular matrix components. The three different isoforms of TGFβ (TGFβ1-3) exert their effects via two receptors (TGFR1 and R2). TGFβ-signalling is mediated through proteins of the SMAD-family which either promote or inhibit TGFβ signals.
In psoriasis keratinocyte hyperproliferation is a disease hallmark. We have therefore studied the expression of TGFβ1-3, TGFR1 and 2, and of SMAD2, 3, 4, 6, and 7 in tissue biopsies of psoriasis lesional and non-lesional skin as well as in skin of healthy persons.
At the margin of untreated psoriasis plaques and of uninvolved skin of the same patient 5 mm punch biopsies were obtained. For control, similar biopsies were taken from the surrounding skin of freshly excized nevi. Biopsies were stored in RNAlater until further use. Thereafter, whole RNA was extracted and subjected to real-time PCR using specific primers.
The results of this investigation showed a significant decrease of TGFβ in lesional and non-lesional psoriasis skin and an increase of TGFβ in psoriasis non-lesional skin. In lesional skin both TGFβ receptors were decreased.SMAD2 and 3 expression was decreased in psoriasis lesional skin only. The most significant decreased was observed for SMAD4, 6, and 7 in lesional psoriasis skin.The results of this study show that not only TGFβ is decreased in psoriasis lesional and non-lesional skin but also its negative regulator SMAD6 and 7 (inhibitor SMADs) and the common SMAD4. These data may lead to the conclusion that TGFβ-mediated inhibition of keratinocyte proliferation is down-regulated in psoriasis lesional skin and may at least in part contribute to the hyperparakeratosis characteristic for the disease.

P114

Calcitonin Gene-Related Peptide (CGRP) and Adrenomedullin (ADM) Modulate Adhesion Molecule Expression in Human Dermal Microvascular Endothelial Cell (HDMEC) by Interacting with Specifc CGRP/ADM Receptors.

T. E. Scholzen[1], M. Fastrich[1], S. Grundmann[1], T. Brzoska[1], T. A. Luger[1]

[1]University of Muenster, Dept. of Dermatology, Ludwig-Boltzmann Institute for Cell- and Immunobiology of the Skin, D-48149 Muenster

The cutaneous sensory neuron-derived neuropeptide CGRP and the related peptide ADM are capable of inducing vasodilatation and potentially of modulating biological functions of epidermal and dermal cells. Effects of CGRP and ADM are mediated through activation of high-affinity G-protein coupled receptors consisting of a receptor-activity modifying protein (RAMP) and a seven-transmembrane domain calcitonin receptor-like receptor (CRLR) with RAMP-1/CRLR as CGRP and RAMP2/CRLR as presumed ADM receptors. We have previously reported that HDMEC express mRNA for CGRP receptor components. In this study we have addressed the hypothesis that endothelial cell adhesion molecule (ICAM-1, VCAM-1) expression in HDMEC transfected with CGRP/ADM receptor components is modulated by CGRP or ADM, respectively. HDMEC isolated from human foreskins or cells of the endothelial cell line HMEC-1 were transfected with cDNA expression vectors for RAMP-1, RAMP-2 and CRLR. Stimulation of HDMEC or HMEC-1 overexpressing R1/CRLR or R2/CRLR with CGRP or ADM (0.01-1000 nM), respectively, resulted in a dose-dependent upregulation of intracellular cAMP indicating the functionality of receptors. Importantly, stimulation of HDMEC cells transfected with R1/CRLR or R2/CRLR with TNF alpha in combination with CGRP or ADM revealed a reduction of the TNF-induced expression of ICAM-1 and VCAM-1. However, neither transfection of HDMEC with the orphan CRLR nor R1 and R2 alone was sufficient to mediate full cAMP induction or reduction of TNFalpha-induced adhesion molecule expression, which confirms the notion that RAMP expression is essential for CGRP/ADM receptor function and pharmacology. Thus, these novel data suggest that CGRP and even more pronounced ADM by activating high-affinity CGRP (R1/CRLR) and ADM (R2/CRLR) receptors resulting in increased intracellular cAMP are capable of antagonizing TNF-induced HDMEC ICAM-1 or VCAM-1 expression, which may be of importance for the regulation leukocyte-endothelial cell interaction during cutaneous neurogenic inflammatory responses.

P115

Stress and Substance P induce premature hair follicle regression (catagen) and mast cell degranulation: indications for local Substance P effects on mast cells in anagen-catagen transformation

A. Peter[1], E. M. Peters[1], B. Handjiski[1], E. Hagen[1], R. Paus[2], P. C. Arck[1]

[1]Charité, Humboldt University, Berlin, Dept. of Internal Medicine, 13353 Berlin, Deutschland
[2]University Hospital Hamburg Eppendorf, Dept. of Dermatology, 20246 Hamburg, Deutschland

It has been much disputed whether or not stress can cause hair loss (telogen effluvium) in a clinically relevant manner. Despite the paramount psychosocial importance of hair in human society, this central, yet enigmatic and controversial problem of clinically applied stress research has not been systematically studied in appropriate animal models. We now show that psychoemotional stress indeed alters actual HF cycling *in vivo*, i.e. prematurely terminates the normal duration of active hair growth (anagen) in mice. Likewise the proteotypic stress-related neuropeptide substance P (SP) also terminates anagen prematurely in non-stressed mice. SP is delivered to the skin by peptidergic nerve fibers. By immunohistochemistry we detected SP-immunoreactive nerve-fibers in the dermis and subcutis of stressed mice in close contact with mast cells. Further, excessive mast cell degranulation and inflammatory events deleterious to the HF are present in the HF environment of stressed mice. This provides the first solid pathophysiological mechanism for how stress may actually cause telogen effluvium, i.e. by hair cycle manipulation and neuroimmunological events that combine to terminate anagen. Furthermore, we show that most of these hair growth-inhibitory effects of stress can be counteracted effectively by co-administration of a specific SP receptor antagonist in stressed mice. This offers the first convincing rationale how stress-induced hair loss in men may be pharmacologically managed effectively.

P116

Arachidonic acid activates the leukotriene inflammatory signaling pathway in SZ95 sebocytes in vitro

T. Alestas[1], W. Chen[2], C. C. Zouboulis[1]

[1]Freie Univesität Berlin, Dermatologie, 12200 Berlin, Deutschland
[2]National Cheng Kung University, Department of Dermatology, 000 Tainan, Taiwan

Acne is a common inflammatory disorder, whereas hormones, bacteria and lipids may play significant roles in its pathogenesis. Sebocytes contain free fatty acids, such as linoleic acid and arachidonic acid (AA), which both can be catalyzed by lipoxygenases (LOX). 5-LOX converts intracellular and extracellular AA to leukotriene A4 (LTA4). LTA4 is further metabolized to LTB4 by the LTA4 hydrolase. Leukotrienes exhibit strong pro-inflammatory properties. LTB4 is a ligand for peroxisome proliferator-activated receptor-α (PPARα) whose regulation affects inflammatory activity and lipid metabolism. In this study, we have investigated the expression of 5-LOX, LTA4 hydrolase and PPARα at the mRNA and protein levels in human SZ95 sebocytes in vitro. 5-LOX and LTA4 hydrolase were detected at the mRNA (RT-PCR) and protein (western blot) levels in SZ95 sebocytes treated with AA and / or calcium ionophore A23187 for 0.5 to 24h. 5-LOX was localized in the cytoplasm by immunocytochemistry. Furthermore, PPARα was expressed at the mRNA level. These experimental data provide evidence that the leukotriene inflammatory signaling pathway can be induced in human sebocytes by AA and support our previous clinical results on the successful administration of a 5-LOX inhibitor in the treatment of inflammatory acne lesions.

P117

Possible role of glial cell line-derived neurotrophic factor (GDNF) and neurturin in human hair follicle biology

M. A. Adly[1], H. A. Assaf[1], P. Petile[2], R. Paus[1]

[1]Univ Hosp Hamburg-Eppendorf, Univ of Hamburg, Dept. of Dermatology, 20246 Hamburg, Deutschland
[2]Cutech Srl, 35129 Padova, Italien

GDNF and another related member of the GDNF family, neurturin (NTN), as well as their cognate receptors (GFRα-1, GFRα-2) are essential for nervous system development. Though we have recently shown that they are also involved in murine hair cycle control (Am J Pathol 156:1041, 2000), their role of GDNF and NTN in human hair biology is still to be elucidated. As a first step towards this end, the immunoreactivity (IR) of GDNF, NTN, GFRα-1, GFRα-2 and their common signal transduction element, c-Ret, were studied in human scalp hair follicles (HF) *in situ* by immunofluorescent and light microscopic immunohistology. All examined antigens showed prominent follicular IR. GDNF IR was strongest in the ORS, IRS, matrix cells, and the sebaceous gland, and less prominent in the DP, of human anagen scalp HF, while it was only weak in telogen HF. GDNF IR was also detected throughout the epidermis, except for the stratum corneum. NTN IR was seen in IRS, matrix cells, DP and connective tissue sheath (CTS) of anagen HF as well as in the papillary dermis. GFRα-1 and GFRα-2 IR were found in the hair matrix, ORS, IRS, DP and/or CTS and in some epidermal cells. c-Ret was restricted to the IRS, ORS mid region and some matrix cells, suggesting that intrafollicular signaltransduction via GDNF/NTN receptors is most relevant in these HF compartments. These observations provide the first indication that GDNF, NTN, GFRα-1, GFRα-2 and c-Ret are expressed also in human skin and suggest that GFRα-mediated signalling plays an important role not only in murine, but also in human HF biology.

P118

Interaction of human hematopoietic stem cells with bacterial pathogens

A. Kolb-Mäurer[1], M. Wilhelm[2], F. Weissinger[2], E. B. Broecker[1], W. Goebel[3]

[1]Universitätsklinik Würzburg, Hautklinik, 97080 Würzburg, Deutschland
[2]Universitätsklinik Würzburg, Med. Poliklinik, D-97070 Würzburg,
[3]Biozentrum Am Hubland, Lehrstuhl fuer Mikrobiologie, 97074 Wuerzburg, Deutschland

Primitive hematopoietic stem cells (HSCs) in the bone marrow are rare pluripotent cells with the capacity to give rise to all lineages of blood cells. During commitment, progenitor cells are comprised mainly of cells with the potential of differentiation in one or two lineages. This commitment involves the acquisition of specific growth factor receptors and the loss of others. Viral and bacterial infections may lead to profound disturbance of hematopoesis which is possibly due to different susceptibility of HSCs to infectious agents. Here we show that these quiescent human HSCs are fully resistant to infection by the intracellular bacteria, Listeria monocytogenes and Salmonella enterica serovar Typhimurium, and extracellular pathogen Yersinia enterocolitica. During myeloid / monocytic differentiation induced by incubation with stem cell factor, thrombopoietin and flt-3 ligand, partially differentiated HSCs emerge which readily take up these pathogens and also latex beads by macropinocytosis. After further monocytic differentiation, bacterial uptake by macropinocytosis still occurs but internalization of the pathogens is now mainly achieved by receptor-mediated phagocytosis. These results suggest that in the case of HSCs, uptake mechanisms for bacteria develop sequentially.

P119

Soluble Factors released by *Pseudomonas aeruginosa*induce a Ca^{2+}-Influx in Keratinocytes via a G-protein linked Pathway

U. Meyer-Hoffert[1], L. Schwichtenberg[1], O. Wiedow[1], J. M. Schröder[1]

[1]Dermatology Department UK Kiel, 24105 Kiel, Deutschland

Keratinocytes are the first cellular line in the host defense against microorganisms like *P. aeruginosa*. They are capable of producing antimicrobial agents like defensins and releasing chemoattractant agents like IL-8 to fight microbial infection. However, little is known about the activation of keratinocytes by pathogen associated molecules (PAMs). After activation many signaling pathways include Ca^{2+}-mobilisation. Therefore the aim of this study was to identify molecules from *P. aeruginosa*, which induce Ca^{2+}-influx in keratinocytes.A high sensitive single cell video imaging system was chosen as a read out system after preliminary results exhibited a fast Ca^{2+}-influx in FURA loaded HaCaT keratinocytes after stimulation with crude supernatants of *P. aeruginosa*. Size exclusion filtration of these supernatants indicated that the Ca^{2+}-inducing activity remained in the >30 kD fraction. Further separation by anion-exchange high performance liquid chromatography exhibited Ca^{2+}-inducing activity at a retention time between 12-14 min. HaCaT cells responded approx. 5 sec after stimulation with an increase of intracellular calcium, which reached normal low calcium levels after approximately 200 sec. In order to investigate heat stability *P. aeruginosa* supernatants were preincubated for 5 min at 70 C, which did not affect the Ca^{2+}-influx. However, incubation for 5 min at 95 C abolished a subsequent Ca^{2+}-influx in HaCaT cells. Preincubation with either pertussis toxin or cholera toxin, two inhibitors of G-protein linked signaling, prevented the Ca^{2+}-influx.Our study demonstrates that keratinocytes are able to recognize soluble factors released by *P. aeruginosa* via a G-protein dependent pathway, which leads to a transient intracellular calcium increase. Further investigations have to identify the molecule structure of the activator and explore the cellular reaction of the keratinocytes to it.

P120

Photodynamic inactivation of MRSA Staphylococcus aureus

T. Maisch[1], X. Feng[2], B. Love[3], R. Szeimies[1], C. Abels[1]

[1]University of Regensburg, Department of Dermatology, D-93042 Regensburg
[2]Solvias AG, CH.4002 Basel, Schweiz
[3]Destiny Pharma, BN1 9SB Brighton, UK

A new approach to treat microbial infections of the skin uses visible or ultraviolet light in combination with a photosensitising dye to induce a phototoxic reaction similar as in photodynamic therapy (PDT) for skin cancer. After uptake of the photosensitizer into bacteria or cells reactive oxygen species (ROS) are generated, which in turn cause oxidative damage. Thus, PDT is able to kill eukaryotic cells as well as bacteria. Therefore, it is important to define appropriate photosensitizers to inactivate bacteria without harming the surrounding tissue *in vivo*. The purpose of this research project is to characterize specific photosensitizers (XF15, XF42 and XF52) for overt toxicity against cultured human skin cells in parallel with the photodynamic efficacy against microorganisms, which are relevant in dermatologic diseases.First of all the serum stability of the photosensitizers was tested. There was no difference regarding the absorption spectra or extinction maxima, no matter whether photosensitizers were resolved in fetal calf serum or not. Different concentrations (0 - 100µM) of photosensitizers as well as different incubation times (5min, 1h or 4h) were used to determine phototoxicity against primary human dermal keratinocytes and human dermal fibroblasts. All tested photosensitizers at a concentration of 0.1 to 10µM exhibit toxicity against both cell types *in vitro* following irradiation ($15.2 mWcm^{-2}$, $13.7 Jcm^{-2}$) after 5 min incubation. Only the incubation with higher concentrations (10µM - 100µM) showed a significant dark toxicity. The antibacterial activity of the photosensitizers were investigated using two gram (+)*S. aureus* strains and one gram (-)*E. coli strain*. Both gram (+)strains were biochemically and for resistance tested: Strain one is a sensitive strain against antibiotics whereas the other one is characterized as a MRSA-strain. Following incubation with 0.01µM irradiation yielded a 2-log step growth reduction (cfu-assay) of both strains with the photosensitizer XF52. At this concentration no toxicity towards fibroblasts was detected. Toxicity occured only after incubation with 0.1µM of XF52. In conclusion, the results show for the first time a therapeutic window *in vitro* to kill a MR *S. aureus* strain by a photodynamic reaction without damage to eukaryotic cells.

P121

Effects of occlusive and semi-occlusive foils on wound healing

M. Schunck[1], C. Neumann[1], J. M. Jensen[1], E. Proksch[1]

[1]Christian-Albrechts-Universitaet, Dermatologie, 24105 Kiel, Deutschland

Semi-occlusive foils are widely used for the treatment of recalcitrant wounds and ulcers. It is claimed that a moist environment beneath occlusive foils promotes wound healing, but, the mechanism of action are only known in part. We investigated the effects of different dressings on wound healing in vivo. Full-thickness wounds 20 mm in diameter were made on the flank skin of hairless mice. Immediately, the wounds were covered with either an occlusive (Latex) or a semi-occlusive foil (Comfeel®) or were left unoccluded. Healing was assessed by computer-assisted planimetrical measurements of wound area. Wound repair was then analysed in detail for the rate of wound contraction and for the rate of re-epithelialization. In addition, we examined the rate of keratinocytes migration by western blotting for STAT-3. This transcription factor has been shown to be a marker of migrating keratinocytes (SANO et al., 1999; EMBO 18 (17), 4657-68). We found that the healing rate was significantly reduced by both dressing materials during the first days of wound healing compared to unoccluded. Wound closure reached 22 % with the Comfeel, 32 % with the Latex foil and 75 % without occlusion within six days after wounding. Complete wound closure occurred within 12 days in unoccluded control and within 16 days in Latex and in Comfeel occluded skin. Wound contraction was significant reduced under dressing conditions in comparison to the untreated control. Also, migration of keratinocytes was delayed by the foils. In occluded mice STAT-3 was expressed later during wound healing than in the unoccluded mice. Compared to the occlusive Latex dressing we found a greater amount of STAT-3 after the use of the semi-occlusive Comfeel foil. Although untreated skin wounds led to faster epithelial regeneration, the final rate of re-epithelialization was increased by Latex (13 %) and Comfeel (16 %) treatments. Latex occlusion led to a significant irritation, which was not observed under the Comfeel foil. We conclude that the semi-occlusive foil Comfeel, possibly by providing an artificial barrier, reduces the speed of initial wound healing, but prevents excessive wound contraction and promotes re-epithelization. The increased STAT-3 level demonstrates, that the Comfeel foil facilitates the cellular migration of keratinocytes.

P122

Altered Gene Expression in Normal Human Epidermal Keratinocytes by Retinoic Acid (RA) Isomers and their 4-oxo-Metabolites: 4-oxo-13cis RA is the most potent derivative

R. Heise[1], G. Zwadlo-Klarwasser[1], H. F. Merk[1], D. R. Bickers[2], F. Jugert[1], J. M. Baron[1]

[1]RWTH Aachen, Hautklinik, 52074 Aachen, Deutschland
[2]Columbia University, Dept of Dermatology, NY New York, USA

Vitamin A and its natural and synthetic derivatives (retinoids) exert profound effects on fundamental processes such as vision, embryonic development and cell proliferation and differentiation. Retinoids are effective in the treatment of numerous dermatologic disorders. In this experiment RA isomers and their 4-oxo metabolites were incubated with cultured NHEK for 24 hours and analysis of differentially expressed genes was performed using DNA-Chip-technology. We examined 4400 sequence-validated cDNAs and ESTs arrayed on DermArray GeneFilters microarray. Incubation of cells with 10-5M of the respective retinoic acid isomer for 24 hours revealed the upregulation of keratin 13, keratin 7 and metallothionein and downregulation of keratin 10 and caseinolytic protease by 13cis-, all-trans- and 9cis- RA. Expression of TIMP1, annexin 13 and collagen XVIII, is downregulated by 13cis and all-trans-RA but upregulated by 9-cis-RA. Expression of MMP7 is downregulated by 13cis and all-trans-RA but expression is not mediated by 9cis-RA. These data reveal that 13cis- and all-trans-RA have a similar effect on the expression of genes related to extracellular matrix compounds and metabolism, whereas 9-cis seems to have the opposite effect. Incubation of cells with 10-5M of the 4-oxo-metabolites for 24 hours revealed similar expression profiles compared to their parent compounds. 4-oxo-13cis- and 4-oxo-all-trans-RA also had a similar effect on the expression of genes related to extracellular matrix compounds and metabolism, whereas 4-oxo-9-cis RA seemed to have the opposite effect. Concerning the number of differentially regulated genes, 4-oxo-13cis RA seems to be the most potent compound. This substance was able to up- or down-regulate more than twice as many genes as the other RA isomers or 4-oxo-metabolites. Interestingly, these 4-oxo metabolites showed a comparable but not completely similar gene expression profile in comparison with their respective parent compound. The three 4 oxo-metabolites show differences in their gene expression profiles suggesting that the stoichiometry of these different compounds may be the decisive element controlling the therapeutic effects of the administered drug.

P123

Pityriarubins A, B and C: novel putative antiinflammatoric agents from*Malassezia furfur*; inhibition of respiratory burst in granulocytes

H. J. Kraemer[1], D. Kessler[2], W. Steglich[3], P. Mayser[2]

[1]Justus-Liebig-Universitaet Giessen, Klin. Pharmakologie, D-35385 Giessen
[2]Justus-Liebig-Universität Giessen, Zentrum fuer Dermatologie und Andrologie, 35385 Giessen, Deutschland
[3]Ludwig-Maximilians-Universitaet Muenchen, Dept. Chemie, D-81377 München

Tryptophan as sole nitrogen source induces the synthesis of fluorochromes and pigments in cultures of *Malassezia (M.) furfur*. In lesions of pityriasis versicolor in contrast to other fungal infections diminuished or no inflammatory reaction of the host occurs. We isolated three metabolites of *M. furfur* (pityriarubins A, B, C), which were chemically identified as bisindolylcyclopentene-1,3-diones. Structurally, these compounds resemble the protein kinase C inhibitor acyriarubin A (bisindolymaleimide). *M. furfur* (CBS1878) was grown for 14 days on a special medium (30ml Tween 80, 3g Trp, 20g Agar dissolved in 1L water) at 30ºC. The agar was extracted with ethyl acetate. The resulting strongly coloured extract was submitted to column chromatography (Sephadex LH20, elution with methanol), followed by preparative TLC (eluent: toluene:ethyl formate:formic acid;10:5:3) and preparative RP-HPLC (linear gradient elution with acetonitrile/water 0-100%; RP8). Three red substances were isolated and structurally elucidated by high-resolution MS and NMR. The pure compounds were used in the burst experiments. Granulocytes were isolated from whole blood of healthy donors. Respiratory burst was initiated by incubation with calcium-ionophore A23187, N-formyl-MET-LEU-PHE (fmlf), 1,2-dioctanoyl-glycerole, phorbol 12-myristate 13-acetate and aluminum fluoride. Release of O_2 radicals was measured by reduction of cytochrome C. Incubation of granulocytes with each pityriarubin A, B, C prior to burst induction revealed dose-dependent inhibition in case of A23 and fmlf. The IC_{50} was determined as 2 µM for all compounds. In case of the other stimuli no inhibition was observed, in contrast to acyriarubin A. We postulate a highly specific influence on the signalling of respiratory burst. The compounds may contribute to the missing granulocyte-activation in vivo.

P124

Inhibition of NF-kB and Induction of Apoptotic Processes in Human Keratinocytes After Treatment with Light Activated Curcuma Longa Extract

J. Dujic[1,2], S. Kippenberger[1], S. Simon[1], A. Ramirez-Bosca[3], J. Bereiter-Hahn[2], R. Kaufmann[1], A. Bernd[1]

[1]JW Goethe Universitaet, Dermatologie, 60590 Frankfrt/M., Deutschland
[2]JW Goethe Universitaet, Arbeitskreis Kinematische Zellforschung, 60439 Frankfurt/M, Deutschland
[3]ASAC Pharmaceutical, 03006 Alicante, Spanien

In previous studies we could show that Curcuma longa extract (ZCL4) combined with both, UVA or visible light induces strong growth inhibition in different cell lines. Now we present data about the underlying mechanisms. Cell membrane damages were studied using the method of LDH release, cell growth was measured by BrdU incorporation, apoptosis was investigated by Hoechst staining, by measuring of the release of Cytochrome C from mitochondria, and by determination of caspases 9 activation using western blotting. The NF-kB activity was measured using a dual luciferase reporter assay. Treatment of cultures of human Keratinocytes (HaCaT) with ZCL4 (1-10 microgram/ml) and UVA (1J/cm²) inhibited the incorporation of BrdU nearly totally without any detectable release of cytoplasmic LDH into the supernatant. The growth inhibition was reversible after treatment with ZCL4 concentrations <5 microgram/ml. From 5 microgram/ml ZCL4 on we found in the presence of UVA an induction of apoptotic processes. Hoechst staining showed an concentration dependent increase of fractionated cell nuclei after treatment with ZCL4 plus UVA. In the absence of UVA the ZCL4 treated cultures contained similar amounts of apoptotic cells as untreated control cultures. The release of cytochrome C from mitochondria and the activation of caspase 9 corresponded very well with the chromatin condensation and the occurrence of fractionated nuclei. At the same time a concentration dependent inhibition of NF-kB activity was detected. Our results show the induction of apoptosis in cell cultures after treatment with Curcuma longa extract combined with UVA irradiation without any sign of cell membrane damages up to 10 microgram/ml ZCL4. The inhibition of the anti-apoptotic nuclear factor kB might contribute to the induction of apoptotic processes after cell treatment with UVA activated Curcuma longa extract. These findings could lead to a new photo therapeutic treatment strategy.

P125

Dimethylfumarate Inhibits Survival of Human Mast Cells

L. M. Preussner[1], U. Mrowietz[2], K. Hartmann[1]

[1]Universitaet zu Koeln, Dermatologie, 50931 Koeln, Deutschland
[2]Universitaet zu Kiel, Dermatologie, 24105 Kiel, Deutschland

Fumaric acid esters, particularly dimethylfumarate (DMF), are used for the treatment of psoriasis. However, the effect of fumaric acid esters on inflammatory cells is not fully understood. In the present study, the effect of fumaric acid esters on proliferation and apoptosis of the human mast cell line HMC-1 and cord blood-derived mast cells (CBMC) was investigated. Proliferation was measured by microscopic counting of trypan blue-negative cells. To assess the number of apoptotic cells, the uptake of PI and binding of annexin-V was investigated by flow cytometry. Immunoblotting was performed to investigate the expression of bcl-2, bax, and bcl-xL protein. DMF, but not methylhydrogenfumarate, methylhydrogenfumarate calcium salt or free fumaric acid, was able to significantly inhibit proliferation and viability of HMC-1 cells. In addition, DMF was shown to induce apoptosis of HMC-1 cells and CBMC in a dose- and time-dependent manner. In HMC-1 cells, apoptosis following incubation with DMF was associated with an increased expression of the pro-apoptotic protein bax and a decreased expression of anti-apoptotic bcl-2 and bcl-xL. Thus, in contrast to other fumaric acid esters, DMF is able to regulate proliferation and apoptosis of human mast cells in vitro. Our observations may in part explain the beneficial effect of DMF in psoriasis and may also stimulate further investigations into the effect of DMF on other inflammatory diseases.

P126

Levels of oxidized arachidonic acid metabolites, IL-1 and IL-6 in microdialysates of human skin and HaCaT-cultures in UVB-erythema and influence of diclofenac (DCF)

J. Grundmann[1,2], I. Wiswedel[2], R. Böckelmann[1], M. Boschmann[3], D. Hirsch[2], C. Müller-Goymann[4], B. Bonnekoh[1], H. Gollnick[1]

[1]University Magdeburg, Dermatology, 39120 Magdeburg, Deutschland
[2]University Magdeburg, Pathobiochemistry, 39120 Magdeburg, Deutschland
[3]Dpt of Nutrition, Biochemistry and Physiology, 14558 Bergholz-Rehbrücke, Deutschland
[4]TU Braunschweig, Pharm Technology, 39120 Braunschweig, Deutschland

Oxidative stress has been implicated in the pathophysiology of several human diseases. Arachidonic acid (AA) as one of the most abundant polyunsaturated fatty acids (PUFA) in cell membranes is susceptible to free radical attack. Besides a lot of secondary oxidation products, radical attack results in the formation of monohydroxyeicosatetraenoic acids (HETEs) and isoprostanes. If HETEs and isoprostanes are formed in significant amounts in membrane-bound phospholipids, they may contribute to modify such important events, as expression of cytokines, apoptosis and vasoconstriction. In current experiments we could show that oxidized AA derivatives, as 5-, 8-12- and 15-HETE, 8-iso-$PGF_{2\alpha}$ and 9α11α-$PGF_{2\alpha}$ were enhanced in microdialysis samples of human skin and HaCaT-cultures following UVB-irradiation, whereas their levels were suppressed by DCF. In further studies we investigated levels of enzymatically formed AA-metabolites (PGE_2 and LTB_4) and interleukins (IL-1 and IL-6) in UVB-irradiated skin and HaCaT-keratinocytes. It could be shown that PGE2 increased in a dose dependent manner after UVB-irradiation in cultured keratinocytes. The response to DCF has to be established in further experiments. In microdialysates PGE_2 levels are very low and hardly to detect by sensitive NICI-GC-MS. LTB4 levels were quantified in microdialysis samples by GC-MS, but they did not significantly respond to UVB and DCF. In HaCaT-cultures the number of viable cells was decreased by UVB up to 57.9 ± 21.0 % of untreated controls. This effect was prevented by 2.5 µM DCF (97.7 ± 31.9 %, $p< 0.02$). UVB induced IL-6 concentrations (487,8 ± 51,1 pg/ml) tended to be lowered by DCF (470,9 ± 68,4 pg/ml). The concentration of IL-1, however, was not changed by DCF in controls and also under UVB exposure. Further investigations may show whether these new findings may also be relevant to validate therapeutical strategies for other inflammatory skin conditions.

P127

All-trans-retinoic-acid treatment leads to increased UV-induced apoptosis in in vitro reconstructed human epidermis

P. Mrass[1], M. Rendl[1], M. Mildner[1], E. Tschachler[1,2]

[1]Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, 1160 Wien, Oesterreich
[2]Centre de Recherches et dÂ´Investigation Epidermiques et Sensorielles (CE.R.I.E.S.), 29521 Neuilly, France

All-trans-retinoic-acid (ATRA)-treatment leads to an upregulation of several pro-apoptotic caspases, and an increased sensitivity to UV-induced apoptosis in primary keratinocytes in monolayer culture (KC). Because retinoids often show different effects in vitro and in vivo, we studied the effect of retinoids in a three-dimensional in vitro reconstruction of human skin. As we have reported previously, ATRA-treatment of skin equivalents (SE) leads to an increase in the level of pro-caspase-3, and the appearance of KC positive for active caspase-3 (10%). Moreover, ATRA-treatment sensitized SE to UV-induced apoptosis, with >60% of KC positive for active caspase-3, after UV-irradiation (30 mJ/cm²). Apoptotic KC were located mainly in suprabasal layers. In comparison, in DMSO-treated SE far less apoptotic KC were observed (10%), which were located predominantly in the basal layer. Because, the p53-dependent apoptotic pathway plays an important role in UV-induced apoptosis, we were interested to see whether ATRA also has an influence on the level of p53 expression. Indeed, we observed that after ATRA-treatment the protein-level of p53 was significantly upregulated in KC in monolayer culture. Consistently, ATRA treated KC were highly susceptible to apoptosis induced by doxorubicin, a substance which induces p53-dependent apoptosis. Cell-death could be blocked completely by the caspase-inhibitor zVAD, and almost completely using the p53-inhibitor alpha-pifithrin. In DMSO-treated SE, p53 was found almost exclusively in basal KC. ATRA-treatment induced p53 in suprabasal levels corresponding to the KC apoptosis observed after UV treatment. However, after UV-irradiation most of the cells expressing p53, were negative for active caspase-3. An explanation for this finding could be, that after p53 has initated the apoptotic machinery, it is destroyed in the dying cells. In conclusion, we show for the first time, that retinoids also sensitize SE to UV-induced apoptosis, a system much closer to the in vivo situation, indicating that the increased sensitivity of ATRA-treated KC to UV-induced apoptosis, could actually play an important role in the treatment of psorasis in vivo.

P128

The lazaroid tirilazad is a new inhibitor of direct and indirect UV-A induced lipid peroxidation in human dermal fibroblasts

J. Dissemond[1], L. A. Schneider[2], M. Wlaschek[2], T. C. Brauns[1], M. Goos[1], K. Scharffetter-Kochanek[2]

[1]Dermatologie, 45122 Essen, Deutschland
[2]Dermatologie, 89081 Ulm, Deutschland

Lipid peroxidation (LPO) caused by oxidative stress within the tissue leads to destruction and dysfunction of cellular membranes. Human dermal fibroblasts within the skin are subject to constant photooxidative stress caused mainly by deeply penetrating UV-A irradiation. Therefore the membrane damage caused by this photooxidative stress might be a major promoter of photoaging and photocarcinogenesis processes initiated and promoted by long-term UV-A exposure of the skin. The oxidative destruction is counterbalanced by a complicated network of enzymatic and non-enzymatic antioxidants creating the skin's line of defense against UV-A induced reactive oxygen species (ROS). The lazaroid trilazad represents a new synthetic group of antioxidants with structural molecular similarity to glucocorticosteroids. We investigated the antioxidative capacity of the lazaroid tirilazad for direct and indirect UV-A induced lipid peroxidation in human dermal fibroblasts.
In a time- and dose kinetic we could show that trilazad incubated fibroblasts are well protected against subsequent UV-A irradiation and show no increase of MDA levels similar to the unirradiated controls. The same applied, when lipid peroxidation was caused chemically by incubation of human dermal fibroblasts with 200 µM Fe3+-citrate and 1 mM ascorbyl phosphate as a model for indirect UV-A induced skin damage. Lysates of fibroblasts treated this way showed a 10-fold increase of MDA levels, whereas pre-incubation with tirilazad resulted in significantly less increase of MDA levels. Furthermore in a comparison to the well established radical scavenger trolox, an a-tocopherol analogon, trilazad offered better protection to the membranes than trolox.
Our results demonstrate for the first time that the lazaroid tirilazad is an effective inhibitor of direct and indirect UV-A induced increase of MDA as a marker for lipid peroxidation in human dermal fibroblasts.

P129

SPT: A new screening test for phototoxicity and phtotoprotection of topical formulations

S. Ekanayake Mudiyanselage[1], P. Elsner[1], J. J. Thiele[1]

[1]Friedrich-Schiller-University, Department of Dermatology, D-07743 Jena, Deutschland

Phototoxicity testing is of crucial relevance for product safety of all topical formulations in dermatology. To date, most standard phototoxicity tests assess single compounds, however, are unable to screen topical formulations. Based on previous studies identifying specific and highly sensitive photooxidation products of skin surface lipids (Squalenemonohydroperoxide, SqmOOH), we developed a simple and fast method for assessing the photoprotection or phototoxicity of topical formulation.
In principle, plates are coated with equal amounts of donor sebum, covered by a thin film of test products and immediately exposed to defined doses of UVA and UVB. Subsequently, lipophilic extracts are analyzed for SqOOH formation by high performance liquid chromatography (HPLC). To test possible protective or phototoxic effects, known sunscreens, antioxidants or phototoxic drugs were screened in this test system.
The results of this study included significant protection by applying vitamin E (2%), C (3%) and a sunscreen (Parsol 1789, 0,1%) in petrolatum (vehicle). The applicability for identifying phototoxic formulations was validated using 12 known phototoxic substances (including doxycycline, oil of Bergamotte, tetracycline, promethazine, rose bengal and others). Furtermore, new formulations including various plant extracts were identified to be phototoxic, contrasting the photoprotective product claim. The latter effect was verified in vivo on human test subjects. It was demonstrated that SPT is a highly sensitive, fast and reliable tool to assess the phototoxicity of dermatological formulations. Importantly, SPT analysis revealed that phototoxicity is not only dependent on single phototoxic compounds, but also on the vehicle/compounded formulation. Thus, SPT based strategies should be introduced in product safety testing.

P130

Squalene monohydroperoxides induce apoptosis in HaCaT cells

W. Oehrl[1], F. Ogawa[1], S. Ekanayake Mudiyanselage[1], M. Hamburger[2], P. Elsner[1], J. J. Thiele[1]

[1]Friedrich-Schiller-University, Department of Dermatology, D-07743 Jena, Deutschland
[2]Friedrich-Schiller-University, Institute of Pharmacy, D-07743 Jena

Squalene is the most abundant, unsaturated skin surface lipid in human sebum. In response to UVA-irradiation squalene readily undergoes photooxidation. The resulting photooxidation products, squalene monohydroperoxides (SqmOOH), have been detected and characterized in UV-irradiated human sebum in vitro and in vivo. Squalen oxidation products are thought to be involved in the pathophysiology of a number of dermatological disorders, such as acne, rosacea, and pityriasis versicolor. However, the effects of SqmOOH on keratinocytes, the primary epidermal target cells of SqmOOH, are so far poorly understood. The present study addresses the question whether squalene monohydroperoxides may lead to changes in intracellular signal transduction processes in keratinocytes and thus play a role in UV-induced skin damage. To investigate this HaCaT cells were exposed to SqmOOH. The activation of intracellular signalling pathways regulating cell growth and differentiation as well as apoptosis were assessed. It was demonstrated that high doses of SqmOOH exhibited cytotoxic effects on HaCaT cells, while low doses promoted cell proliferation. It was further demonstrated that SqmOOH induce apoptosis in HaCaT keratinocytes by activating a mitochondrial apoptosis pathway involving Caspase 9 and its effector Caspase, Caspase 3 in a time and dose dependent manner. The study further demonstrates that MAPK pathways are differentially induced by SqmOOH, with p38 MAPK being the predominant isoform activated. Furthermore, the use of MAPK inhibitors suggested that MAPKs are involved in induction of apoptosis in response to SqmOOH in HaCaT cells. In conclusion, SqmOOH lead to the induction of a regulated stress response in HaCaT keratinocytes and may therefore contribute to UV-induced skin damage.

P131

Higher DNA repair efficiency in keratinocytes is mediated through induction of excision repair pathways via p53 and its downstream effectors p48, GADD45, and β-polymerase

C. Chung[1], G. Z. Li[1], B. A. Gilchrest[1], T. M. Ruenger[1]

[1]Boston University School of Medicine, Dept. of Dermatology, MA 02118 Boston, USA

We recently reported that primary human keratinocytes repair DNA photoproducts and oxidative DNA damage 2 - 12-fold more efficiently than primary fibroblasts or melanocytes. This difference was demonstrated in cells from 5 - 17 different donors, including all three cell types from one individual donor. Additionally, it was independent of keratinocyte differentiation, as different Ca-concentrations did not alter the high repair efficiency in cultured keratinocytes. In order to elucidate the underlying molecular mechanisms of this difference in DNA repair efficiency, we first determined levels of several DNA repair proteins of the nucleotide- and base-excision repair pathways (NER and BER: XPA, ERCC1, hOGG1, and APE-1) in extracts of primary keratinocytes, fibroblasts, and melanocytes from one donor. No significant differences in expresssion were found. In contrast, a very strong expression of p53 and of activated p53 (serine^{15}-phosphorylated p53) was seen in keratinocytes, while fibroblasts or melanocytes exhibited only weak baseline expression of p53 or activated p53. The high expression of p53 and serine^{15}-p53 was independent of keratinocyte differentiation, as it was seen with high (1.8 mM), intermediate (0.15 mM), and low (0.05 mM) calcium concentrations. It provides a very good explanation why both DNA repair pathways, NER of photoproducts and BER of oxidative DNA damage, are more efficient in keratinocytes, because in recent years, p53 has been identified to induce both global genome-NER and BER. The effect of p53 on NER is mediated by transcriptionally inducing p48XPE and GADD45, and the effect on BER by inducing β-polymerase. Consistent with these effects, we found the protein levels of all these three proteins to be higher in keratinocytes, as compared to fibroblasts or melanocytes. To conclude, it appears that keratinocytes have adapted to their higher exposure to environmental mutagens by upregulating excision repair pathways through p53 and its downstream effectors.

P132

Photodynamic therapy of HaCaT cells with several photosensitizers

U. Hipler[1], A. Radestock[1], D. Winter[1], P. Elsner[1]

[1]Klinik für Dermatologie und dermatologische Allergologie, D-07740 Jena

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, BLC 1010, BLC 1041) 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,Biolitec AG Jena, Germany). Biostimulation and bioinhibition were determined in human immortalized keratinocytes (HaCaT) 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 HaCaT cells with 0.8 J /cm^2 and 12.5 J /cm^2 of 662 nm irradiation after incubation with serveral photosensitizers in different concentrations.Results: Cytotoxcic 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}BLC 1010 (0.19 - 2.28 µM), ED_{50} BLC 1041 (0.21 - 2.44 µM), ED_{50}Chlorin e6 (0.21 - 1.74 µM).Conclusions : The photosensitizers Chlorin e6, BLC 1010 and BLC 1041 are suitable for photodynamic therapy of HaCaT keratinocytes using a low energy laser with an energy density of 12.5 J /cm^2.

P133

Induction of UVB-mediated tolerance does not only induce regulatory T cells but also T effector cells

Y. Aragane[1], A. Maeda[1], K. Ueno[1], F. Yamazaki[1], T. Tezuka[1], T. Schwarz[2]

[1]Kinki University, Department of Dermatolog, 589-8511 Osaka, Japan
[2]University Muenster, Department of Dermatology, 48149 Muenster, Deutschland

Epicutaneous application of haptens induces T effector cells preferentially of the CD8 type which mediate contact hypersensitivity (CHS). Sensitization, however, does not only result in the induction of T effector cells but also of suppressor/regulatory T cells (Tr) belonging to the CD4 type since depletion of CD4 results in an exaggerated CHS response. UVB light induces tolerance via the induction of Tr. Since sensitization induces both T effector cells and Tr we asked whether induction of tolerance by UVB does not only induce Tr but also T effector cells. Thus, C3H/HeN mice were tolerized by painting dinitrofluorobenzene (DNFB) onto UVB-exposed skin. In addition, we utilized a soluble form of the protein Dectin-2 (sDec2). Dectin-2 is a costimulatory molecule expressed on dendritic cells which appears to play a pivotal role in UVB-induced tolerance since injection of sDec2 inhibits the development of UVB-induced tolerance. sDec2 binds to UVB-induced Tr since depletion of sDec2-bound T cells obtained from UVB-tolerized mice results in the loss of transfer of suppression. FACS analysis of T cells obtained from UVB-tolerized mice using a biotinylated form of sDec2 and antibodies against CD4 and CD25 revealed that sDec2 only bound to CD4+ T cells. sDec2-bound CD4+ T cells were mainly positive for CD25. T cells from UVB-tolerized mice were fractionated into sDec2-bound or -unbound fractions and injected i.v. into naive syngeneic mice. Recipients receiving sDec2-bound T cells could not be sensitized against DNFB, indicating that sDec2-bound T cells transfer tolerance. Recipients receiving sDec2-unbound T cells could be normally sensitized. Even more importantly, a specific ear swelling response could be induced in the recipients of sDec2-unbound T cells when the mice were not sensitized but only challenged with DNFB. The fact that a specific ear swelling response was achieved in the absence of sensitization indicates that the fraction of sDec2-unbound T cells obtained from UVB-tolerized mice must contain DNFB-specific T effector cells. Together, the data indicate that comparable to sensitization which results in the induction of both T effector cells and Tr, induction of tolerance by UVB does not only induce Tr but also induces T effector cells.

P134

Singlet oxygen-induced inhibition of growth factor signaling in dermal fibroblasts

S. M. Schieke[1], J. Krutmann[1], A. Timmer[1], S. Grether-Beck[1], H. Sies[2], N. J. Holbrook[3], L. O. Klotz[2]

[1]Institut für Umweltmedizinische Forschung (IUF), an der Heinrich-Heine-Universität, 40225 Düsseldorf, Deutschland
[2]Institut für Physiologische Chemie I, Heinrich-Heine-Universität, 40225 Düsseldorf, Deutschland
[3]Yale University School of Medicine, Section of Geriatrics, Dept. of Internal Medicine, 06520-8025 New Haven, USA

Singlet oxygen, an electronically excited form of molecular oxygen, can be generated photochemically, such as in photodynamic therapy, or during exposure to ultraviolet radiation, or metabolically in inflamed tissues. It is a primary mediator of the activation of stress-activated protein kinases (JNK and p38-MAPK) elicited by ultraviolet A (UVA; 320-400 nm). In the present study the effects of singlet oxygen on growth factor-induced activation of survival pathways, i.e. the extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B (PKB)/Akt pathways, were analysed in human dermal fibroblasts. The activation of ERK 1/2 and Akt as induced by stimulation with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) was inhibited by singlet oxygen generated intracellularly upon photoexcitation of Rose Bengal. Thus, singlet oxygen is capable of attenuating the activation of cellular survival pathways by growth factors such as EGF and PDGF. Since photodynamic therapy (PDT)-induced apoptosis is associated with increased formation of ceramides we evaluated the role of ceramides, as potential mediators of the survival pathway inhibition by singlet oxygen. Indeed, both UVA and singlet oxygen lead to ceramide generation in human skin cells, and the attenuation of EGF- and PDGF-induced activation of ERK 1/2- and Akt by singlet oxygen was mimicked by stimulation of fibroblasts with the cell-permeable C_2-ceramide. This points to a role of ceramides as mediators of the singlet oxygen-induced inhibition of growth factor-induced activation of cellular survival pathways and to a novel mechanism of singlet oxygen toxicity.

P135

Investigation of the photooxidative reactions following photosensitization with Photofrin® *in vitro*

K. Scherer[1], W. Bäumler[1], C. Abels[1]

[1]Universität Regensburg, Klinik und Poliklinik für Dermatologie, 93042 Regensburg, Deutschland

Activation of porphyrins by light generates either oxygen radicals (type I-reaction) and/or singlet oxygen (type II-reaction). The generated reactive oxygen species (ROS) induce cell death or modulation of cellular functions. Phototoxicity was correlated with the subcellular localization of porphyrin-based photosensitizer Photofrin, amount of oxidated fatty acids and cytokine-levels. HT29 (human colonic cancer cells) were incubated with Photofrin (0-20 µg/ml, D2O) for 90 min or 24 h and irradiated (590-750 nm, 24 J/cm^2, 40 mW/cm^2). Subcellular localization of the photosensitizer was determined by fluorescence microscopy and costaining with Lyso-Tracker Green (lysosomes) and Rhodamine 6G (mitochondria). Membrane damage was determined by measuring lipidperoxidation products: lipidperoxides (TBARS) and lipidhydroperoxides (LOOH). Different quenchers of ROS, e.g. NaN_3, histidine (5-100 mM/l) or mannitol (50 mM/ml), elucidated the extent of photooxidation-types involved (type I or type II). The concentration of IL-1α and TGF-β induced was assessed by purchasable ELISAs. Photofrin localizes in the plasma membrane after 90 min and in intracellular membranes after 24 h. TBARS and LOOH increase up to a sensitizer concentration of 15 µg/ml independently from the subcellular localization. Il-1α- and TGF-β-levels increase up to 15-fold at 2.5 µg/ml Photofrin where no lipidperoxidaton-products can be measured and independently of the subcellular localization. Quenching singlet oxygen and quenching of the hydroxyl radical leads to an increase in cell viability and cytokine-levels, but results in decrease of both lipidperoxidation products. However, quenching of the hydroxyl radical increases the cytokine-levels and decreases only LOOH, but not TBARS. These data suggest the involvement of both reaction types following Photofrin-photosensitization. However, extensive involvement of the type-I reaction can only be seen when assessing the quenching performance of the early reaction products LOOH, not with later formed and more commonly assessed TBARS or cell viability. Thus, to obtain valid statements on the type of photooxidation it is not enough to assess the influence of quenching on cell viability. In addition, if quenching reduces effectively the generation of ROS and thus decreases lipidperoxidation a marked increase of cytokines can be shown.

P136

The singlet oxygen generation at different oxygen concentrations

W. Bäumler[1], K. Scherer[1], D. Baumer[2], M. Maier[2], R. Engl[1]

[1]Universität Regensburg, Klinik und Poliklinik für Dermatologie, 93042 Regensburg, Deutschland
[2]Universität Regensburg, Institut für experimentelle und angewandte Physik, 93040 Regensburg, Deutschland

Based on numerous experiments *in vitro*, singlet oxygen is considered to play a major role in photooxidative processes during PUVA or photodynamic therapy (PDT). However, the generation efficacy of singlet oxygen depends critically on the respective oxygen partial pressure which is significantly different for the situation in vitro (about 140 Torr) and in vivo (less than 20 Torr in the skin). Therefore, the generation of singlet oxygen by the linear furocoumarin 8-methoxypsoralen (8-MOP) and the photosensitizer 9-acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene (ATMPn) has been investigated in solution at an oxygen concentration ranging from 0 to maximal 0.5 mM (0 - 280 Torr). Using a highly sensitive infrared photomultiplier, the complete time dependence of singlet oxygen luminescence was measured at 1270 nm. The respective relaxation rates and rate constants were determined, in particular at different oxygen concentrations. The oxygen concentration dependence of the singlet oxygen quantum yield (Φ_Δ) were calculated. The results show that Φ_Δ decreased significantly with decreasing oxygen concentration. The completely different oxygen concentrations i*n vitro a*nd in*vivo*lead therefore to a different quantum yield of singlet oxygen generation in both situations. Thus, one has to be careful when transferring results from i*n vitro*experiments to PUVA or PDT i*n vivo,* in particular regarding the role of singlet oxygen (type-II reaction) as compared to the type-I reaction.

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Singlet oxygen proven directly by its luminescence in cells

K. Scherer[1], R. Engl[1], W. Bäumler[1], H. Stockmeier[1], S. Habecker[1], C. Abels[1], M. Maier[2]

[1]Universität Regensburg, Klinik und Poliklinik für Dermatologie, 93042 Regensburg, Deutschland
[2]Universität Regensburg, Institut für experimentelle und angewandte Physik, 93040 Regensburg, Deutschland

The generation of singlet oxygen is the first step of an oxidative damage of cellular constituents leading to the activation or even to the killing of cells. Singlet oxygen is usually detected by using enhancers (D_2O) or quenchers (e.g. sodium azide) of its cellular effects. However, these indirect methods are invasive and frequently yield no unequivocal results. In the present study singlet oxygen was detected directly by the time-resolved measurement of its luminescence.Singlet oxygen was generated by Photofrin which accumulates in cellular membranes. Since phospholipids are main constituents of cellular membranes, the decay time of singlet oxygen luminescence in phosphatidylcholine droplets in aqueous suspensions was measured for the first time, yielding 16 ± 4 µs. The same decay time was measured when singlet oxygen was generated by Photofrin in the plasma membrane or in the mitochondrial membranes of human colonic cancer cells (HT29) which were suspended in H_2O or D_2O. The results provide clear evidence that the direct detection of singlet oxygen by its luminescence in cells is possible. Cell viability data show that the luminescence measurement is non-invasive up to a high Photofrin concentration. Only at very high Photofrin concentrations the cells were damaged to some extent, which can be prevented by adding the quencher sodium azide or histidine. In contrast, using D_2O, there was no enhancement of the damage by singlet oxygen generated in the mitochondrial membranes and therefore the indirect detection of singlet oxygen yields an inconsistent result. The latter might be true also for other experiments when singlet oxygen is generated in subcellular localizations of no or little access to D_2O.Thus, the direct detection of singlet oxygen by its luminescence enables a non-invasive and detailed insight into the primary mechanisms of interaction between singlet oxygen and cellular compartments.

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The Osmolyte Strategy of Human Keratinocytes

J. Krutmann[1], U. Warskulat[2], A. Reinen[2], S. Grether-Beck[1], D. Häussinger[2]

[1]Institut für umweltmedizinische Forschung (IUF) an der Heinrich-Heine-Universität GmbH, 40225 Düsseldorf, Deutschland
[2]Department of Gastroenterology, Hepatology and Infectiology, Heinrich-Heine-Universität, 40225 Düsseldorf, Deutschland

Compatible organic osmolytes, such as betaine, myo-inositol and taurine, are specifically accumulated or released in response to hyperosmotic cell shrinkage or hypoosmotic cell swelling, respectively, to maintain cell volume homeostasis. In addition, osmolyte uptake is important for cell protection, e.g. against oxidative stress. This so called osmolyte strategy requires the expression of specific osmolyte transporting systems such as the betaine-/γ-amino-n-butyric acid transporter BGT-1, the sodium-dependent myoinositiol transporter SMIT and the taurine transporter TAUT. Osmolyte strategies have been characterized in great detail in liver, kidney and neural cells and found to be tissue-specific. In marked contrast, nothing is known about osmolytes in the skin. Here we report that primary normal human keratinocytes (NHK) express mRNA specific for BGT-1, SMIT and TAUT. In comparison to normoosmotic (305 mosmol/l) controls, which only expressed TAUT mRNA, significant induction of BGT-1 and SMIT mRNA expression was observed 6h and 24h after hyperosmotic exposure (405 mosmol/l). Expression of osmolyte transporters was associated with an increased uptake of radiolabeled osmolytes. Conversely, hypoosomotic (205 mosmol/l) stimulation induced a significant efflux of osmolytes. Essentially identical data were obtained in HaCaT cells. Cellular hydration can change after oxidative stress. Since UVBR is a major source of oxidative stress and since UVBR and osmotic stimulation induce similar signaling events we next assessed the influence of UVBR on osmolyte transport. Exposure to UVBR (0-100 J/cm^2) significantly stimulated myo-inositol and taurine uptake in NHK and taurine and betaine uptake as well as mRNA expression for BGT-1 and TAUT in HaCaT cells. Osmolyte efflux was not affected by UVBR in either cell type. These studies for the first time demonstrate that NHK possess an osmolyte strategy, which is important for their capacity to maintain cell volume homeostasis and seems to be part of their response to UVBR.

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Keratin5-Cre/LoxP mediated inactivation of Vascular Endothelial Growth Factor sensitizes Mouse skin to UVB-induced photo-damage

C. Barresi[1], H. Rossiter[1], E. F. Wagner[2], E. Tschachler[1,3]

[1]AKH, Immundermatologie, 1090 Wien, Oesterreich
[2]I.M.P., 1030 Wien, Oesterreich
[3]CE.R.I.E.S., 29521 Neuilly, Frankreich

Excessive exposure of skin to sunlight results in erythema, dilation of dermal blood vessels and vascular hyperpermeability, suggesting that 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 (termed K5/Cre VEGF flox/flox mice), and used these animals to study the contribution of KC-derived VEGF to acute UVB-induced photodamage of skin. We find that these mice develop burn-like lesions after a single UVB irradiation, at a dose at which the control mice are macroscopically completely unaffected. At higher doses, at which the control mice also show macroscopic evidence of acute photo-damage, the K5/Cre VEGF flox/flox mice develop the lesions earlier, and they heal more slowly than in the control mice. Microscopic examination of the irradiated skin revealed massive inflammation, with complete loss of the epidermis in the mutant but not in the control mice. In order to identify the mechanism by which VEGF could contribute to protection against UVB-induced photodamage, we have so far estimated numbers of apoptotic cells in dermis and epidermis, the dermal blood vessel density as well as the numbers of proliferating KC, since UVB irradiation leads to epidermal hyperplasia. Interestingly, all of these parameters were similar in both mutant and control mice. We conclude that in the absence of functional VEGF in epidermal KC skin is predisposed to exreme sensitivity to UVB-induced photodamage. The mechanism however appears to be more complex than either a direct protection of KC against apoptosis or a defect in the survival of the dermal vasculature.

P140

Rapid replication arrest in response to psoralen plus ultraviolet A radiation involves ATM and Cdc25A.

C. Joerges[1], I. Kuntze[1], T. Herzinger[1]

[1]Ludwig-Maximilians-Universität München, Klinik und Poliklinik für Dermatologie und Allergologie, 80337 München, Deutschland

In response to different forms of DNA damage, cell cycle progression is halted at the transition from G1 into S-phase, during replication and/or at the G2/M transition, thereby preventing replication of damaged DNA templates or segregation of broken chromosomes, respectively. Induction of interstrand crosslinks (ICLs) in chromosomal DNA is considered a major reason for the anti-proliferative effect of psoralen plus ultraviolet A (PUVA), but the underlying molecular mechanisms are poorly characterized. We demonstrate sow that HaCaT keratinocytes treated with PUVA arrest with exclusively in S-phase but not at G1/S or G2/M, suggesting that ongoing replication is needed for the registration of ICLs. PUVA induced rapid phosphorylation of the Chk1 checkpoint kinase at Ser345 and a concomitant decrease of Cdc25A, a critical promoter of S-phase progression. The decrease of Cdc25A levels and the S-phase arrest were both abolished by caffeine, suggesting that active checkpoint signaling rather than mechanical blockage by ICLs causes the PUVA induced replication arrest. Abrogation of the arrest by caffeine further suggested the involvement of the caffeine-sensitive checkpoint proteins ATM and/or ATR. Cells lacking Atm or over-expressing Cdc25A temporarily override the PUVA induced S-phase arrest, suggesting the existence of a second, more slowly responding caffeine-sensitive checkpoint pathway.

P141

Antioxidant treatment modulates UVA-, UVB- or ionising irradiation-induced effects on cell cycle

U. Kerkmann[1], K. Gilbertz[2], S. Gaube[1], B. Przybilla[1], M. Placzek[1]

[1]Klinik und Poliklinik für Dermatologie und Allergologie - Innenstadt, 80337 München, Deutschland
[2]Institut für Radiobiologie der Bundeswehr, 80997 München, Deutschland

Irradiation of human cells causes a time dependent decrease of proliferation activity. One important component of the cellular irradiation response is activation of the cell cycle checkpoints. It is known that UV as well as ionising irradiation can activate checkpoints at G1/S, G2/M and within S-phase, but until now it is not clear whether different wavelengths have different effects on cell cycle. Furthermore, we do not know if antioxidants, such as ascorbic acid and Trolox (water soluble vitamin E derivate) can influence these effects.For this reason we irradiated IPC-298 melanoma cell cultures with 10 J/cm2 UVA-, 40 mJ/cm2 UVB- and ionising radiation (0-10 Gy). Irradiation is followed immediately by a 10 min incubation with 10 mM BrdU. We treated cell cultures with antioxidants before and after irradiation. BrdU is a proliferation marker incorporated during S-phase of cycling cells and can therefore be used to discriminate cell cycle progression in each single G1, S or G2 and M phase after irradiation. At 0h, 6h, 12h and 24h after irradiation the cells were fixed and DNA content and BrdU incorporation per cell were analysed by dual parameter flow cytometry.UVB radiation induced an arrest in G1 as well as in G2 and S-phase progression was reduced. UVA-irradiated cells were arrested in G2 but not in G1 and also showed a reduced S-phase progression. Ionising irradiation induced an arrest in G2. Cells were not arrested in G1 or in S and there were no effects of antioxidants evident. Antioxidants enhance the G2 arrest in UVA- and ionising irradiation-treated cells and the lower progression of S-phase cells after UVA-irradiation. These results show that different wavelengths induce different effects on cell cycle. Antioxidants can modulate these effects, especially in UVA- and ionising irradiation-treated cells. Combined treatment extends cell cycle by increasing the arrest at the G2/M checkpoint and provides time for DNA repair. The risk of induction of mutations is therefore reduced.

P142

Induction of photoaging-associated mitochondrial DNA mutations by repetitive UVB irradiation in normal human keratinocytes

M. Berneburg[1], T. Gremmel[1,2], T. Ruzicka[1], J. Krutmann[2]

[1]Molekulare Onkologie und Alterung, Hautklinik, Heinrich-Heine-Universität, 40225 Düssseldorf, Deutschland
[2]Insitut für Umweltmedizinische Forschung, Heinrich-Heine-Universität, 40225 Düsseldorf, Deutschland

Mutations of mitochondrial (mt) DNA play a role in the process of photoaging. The most frequent mutation of the mtDNA, the so called common deletion (CD), which is considered to be a marker for mtDNA mutations, has been reported to be induced in-vivo in the dermal compartment of normal human skin as well as in-vitro in normal human fibroblasts by repetitive exposure to ultraviolet (UV) A radiation. Since it is known that pathways relevant for the process of photoaging can also be induced by UVB radiation in normal human keratinocytes, we asked the question whether low dose repetitive UVB exposure of these cells also leads to the generation of the CD. To determine sublethal conditions for repetitive in-vitro UVB radiation exposure, foreskin-derived normal human keratinocytes were irradiated with doses of UVB radiation ranging from 0, 3, 6, 12, to 24 mJ/cm² three times daily at four consecutive days per week. Cells exposed to 12 and 24 mJ/cm² UVB showed a significant decrease in cell number and viability whereas doses of 6 mJ/cm² and below did not affect the viability of irradiated cells. In the following experiments cells were therefore repetitively exposed to 6mJ/cm² of UVB radiation. The presence of the CD was determined by semiquantitative nested PCR and subsequent Phosphorimager analysis after each week of repetitive UVB exposure. Repetitive irradiation of normal human keratinocytes three times daily, four times per week with 6mJ/cm² of UVB lead to the induction of the CD after the first week of UV exposure with signal intensities increasing up to the 3 weeks timepoint. These stduies for the first time demonstrate that the CD can be induced in normal human keratinocytes by repetitive UVB irradiation. This observation indicates that mt DNA mutations are not only involved in the process of photoaging induced by UVA but also by UVB radiation.

P143

Adhesion Behaviour of Human Keratinocytes is Triggered by Activation of the β 4 Integrin

S. Kippenberger[1], J. Müller[1], M. Guschel[1], R. Kaufmann[1], A. Bernd[1]

[1]Klinikum der Johann Wolfgang Goethe Universität, Department of Dermatology and Venerology, 60590 Frankfurt, Deutschland

Carcinogenesis is considered as a multistep process involving functional changes in the hemidesmosomal organization. In normal skin keratinocytes, expression of the α6β4 integrin is restricted to the proliferative basal layer and mediates stable adhesion to the underlying basement membrane. Observations in carcinoma cells show a functional and spatial dissociation of the α6β4 integrin from the hemidesmosomal complex which stimulates cell migration and therefore, may contribute to carcinoma invasion. We now have evaluated the adhesion behavior of epithelial cells at different stages of transformation in response to activation of the β4 integrin. It is demonstrated that activation of the β4 integrin augmented adhesion of carcinoma and pre-carcinoma cells to non-modified plastic. In contrast adhesion behavior of normal human keratinocytes was not influenced by activation of the β4 integrin. In order to explain the mechanism of β4-mediated adhesion, the hypothesis of an inside-out activation of integrins was tested. Evidence is given that activation of the β4 integrin increased the substrate avidity of the β1 integrin. Furthermore, activation of the β4 integrin led to phosphorylation of PKB/Akt at both phosphorylation sites (Ser473, Thr308). Functional blocking of PKB/Akt by dominant-negative overexpression decreased the substrate affinity in response to β4 integrin activation. These results connect the anti-apoptotic properties of the PKB/Akt signaling cascade to alterations in adhesion behavior, protecting the cells from anoikis. Taken together, the present data establish a link between the activation of the β4 integrin and the substrate affinity of the β1 integrin in carcinoma and pre-carcinoma cells. Hence, these findings provide further insight into the conversion processes during carcinogenesis and show the β4 integrin to be a key regulator of cellular adhesion.

P144

C-KIT-expression in primary cutaneous T-cell lymphoma

T. C. Brauns[1], T. Schultewolter[1], J. Dissemond[1], M. Goos[1]

[1]Universitätsklinik Essen, Hautklinik, 45122 Essen, Deutschland

Background:
Mutations of the stem cell factor receptor C-KIT play a major pathogenetic role in the development of different malignant diseases like human mastocytosis, myeloproliferative disorders, gastrointestinal stromal tumors, acute myelogenous leukaemia and sinonasal lymphomas. Further the expression of C-KIT has been described in Hodgkin´s Disease and CD30 positive anaplastic large cell lymphomas. Since it is possible to inhibit C-KIT by innovative kinase-Inhibitors like STI571 it may be an attractive target for new therapeutical approaches. Therefore we screened more than 50 different types of cutaneous T-cell lymphomas for the presence of C-KIT

Methods:
Immunhistochemical stainings were performed on paraffin-embedded tissue sections using a polyclonal rabbit anti human C-KIT antibody (DAKO Code No. A4502). Chloresterase-controlstainings were performed on every positive sample to distinguish c-kit positive lymphoma cells from c-kit positive mast cells.

Results:
We found weak expression of C-KIT in 7 of 21 patients with primary cutaneous CD30 positive anaplastic large cell lymphoma, 2 of 8 patients with primary cutaneous pleomorphic T-cell lymphoma, 8 of 19 patients suffering from mycosis fungoides and 3 of 5 patients with Sezary´s syndrome. Generally only a very small population of the lymphoma-cells expressed C-KIT. This finding indicates a difference to the systemic variant of CD30 positive anaplastic large cell lymphoma. The potential use of C-KIT-targeting new therapeutical approaches is therefore discussed critically since C-KIT expression is very weak in all investigated types of primary cutaneous lymphoma.

P145

Downregulation of CtBP expression in malignant melanomas leads to induction of MIA expression

I. Poser[1], M. Golob[1], R. Buettner[2], A. Bosserhoff[1]

[1]University Hospital Regensburg, Institute of Pathology, 93053 Regensburg, Deutschland
[2]University Hospital Bonn, Institute of Pathology, 53127 Bonn, Deutschland

Malignant transformation of melanocytes to melanoma cells closely parallels activation of MIA expression and involves a promoter region which we previously referred to as HCR (highly conserved region). The HCR element interacts with the transcription factor MATF ("melanoma associated transcription factor") and confers strong activation of the promoter. Further, mutation and deletion studies described in this study revealed that the permissive site for cell specific promoter activity was located directly 5' to the HCR region. Changes in the DNA sequence 5' adjacent to the MATF binding site led to an MIA promoter activity in benign melanocytes and non-melanocytic cells which usually do not express MIA. Detailed analysis revealed binding of TCF family transcription factors to the repressor element. As this family is known to interact with C-terminal binding protein we explored the role of CtBP1 in silencing MIA gene expression. By reporter gene analysis we determined a strict negative regulation of MIA promoter activity in melanoma cells by CtBP1. Further, we observed strong expression of CtBP1 in primary melanocytes, but a loss of wildtype CtBP1 expression in malignant melanoma *in vitro* and *in vivo*. Therefore, we speculate that CtBP1 has an important negative role in MIA regulation and loss of CtBP1 is implicated in melanoma progression.

P146

Expression of Src-Type Tyrosine Kinases in Cutaneous T CellLymphoma (CTCL).

U. Döbbeling[1], J. Kamarashev[1], R. Dummer[1], G. Burg[1]

[1]Universitaetsspital Zuerich, Dermatologische Klinik, CH-8091 Zuerich, Schweiz

Mycosis fungoides (MF) and its leukemic variant Sézary syndrome (SS) are the most frequent types of cutaneous T cell lymphomas (CTCL) and their aetiology is quite unknown. We recently found that CTCL cells contain constitutive NFkB activities, which could be down-regulated by agents that inhibit src-type tyrosine kinases. The src gene family contains members that mediate T-cell receptor signals and known
oncogenes.
We therefore investigated MF and SS cell lines and skin lesions for src-type tyrosine kinase expression by Western blotting and Immunohistochemistry respectively. Western blot experiments with antibodies specific for c-src, c-fgr, c-yes, lck, and fyn showed that c-src, c-yes, and fyn were expressed in all four tested cell lines. The T-cell receptor associated src-type kinase lck was expressed only in one cell line, indicating that this protein was not involved in NFkB activation in MF and SS cell lines. The c-fgr gene was expressed in no cell line. When we tested MF and SS skin lesions for the expression of the c-src and c-yes oncogenes, we found that c-src was already expressed in early (patch/plaque) stages, whereas c-yes was only expressed in late (tumor) stages.
Our results show that the c-src and c-yes tyrosine kinases, which are not expressed in normal T cells, are present in MF and SS cells. Since src-type tyrosine kinase inhibitors like herbimycin A and PP2 inhibit the constitutive NFkB activities, we suppose that c-src and c-yes are involved in constitutive activation of NFkB in MF and SS cells, which in turn may lead to the expression of further genes that promote the cancerogenesis of CTCL.

P147

Cell type-specific expression of CD95 ligand in human melanoma cell lines using a tyrosinase-derived promoter

L. F. Fecker[1], J. Eberle[1], A. M. Hossini[1], D. L. Bartlett[2], C. E. Orfanos[1], C. C. Geilen[1]

[1]University Medical Center Benjamin Franklin, The Free University of Berlin, Department of Dermatology, 14195 Berlin, Deutschland
[2]National Cancer Institute, National Institutes of Health, Clinical Sciences Division, Surgery Branch and Medicine Branch, MD20892 Bethesda, USA

Malignant melanoma shows only limited response to chemotherapeutic drugs which may be also related to deficient apoptotic mechanisms. In a previous study, we have shown, that apoptosis could be efficiently triggered in human melanoma cell lines in vitro and in nude mice by the expression of CD95 (Fas) ligand. Targeted gene expression is a critical prerequisite when thinking of the therapeutical use of suicide and proapoptotic genes. Tyrosinase-derived promoters seem to be best suited for melanoma because of the high and restricted expression of tyrosinase in pigment cells. For specific expression, we used a tyrosinase-derived promoter, consisting of the human basal element and two tandem upstream murine enhancer elements, described in a previous study. Relative promoter activities were studied after transient transfection of luciferase reporter gene constructs with either the tyrosinase promoter or the constitutive SV-40 promoter. In the two pigmented melanoma cell lines SK-Mel-13 and Mel-2a, the tyrosinase promoter was as strong as the SV-40 promoter or 4.5-times stronger, respectively. On the other hand, in the almost amelanotic cell line A-375, the transfected tyrosinase promoter was only 5% as strong as the SV-40 promoter, and it showed less than 2% of the activity of the SV-40 promoter in the mammary carcinoma cell lines MCF-7 and ZR-75-1 as well as in the neuroectodermal tumor cell line PFSK-1, used as controls. The tyrosinase-derived promoter was then subcloned in front of a full length CD95L cDNA, and apoptosis was quantified 2 days after transient transfection by ELISA. All melanoma cells, analysed, showed significantly elevated apoptosis after transfection of the tyrosinase promoter/CD95L construct, inclusive A-375. The nonmelanoma cell lines showed no response or the response was weaker (MCF-7).These data may demonstrate that pigment gene promoters may be used for targeted expression of proapoptotic genes in melanoma cells. Some apoptosis determined also in nonmelanoma cell lines may be due to the high copy numbers used and to the high proapoptotic potential of transfected CD95L cDNA.

P148

Prognostic value of clinical and immunohistochemical features of Merkel cell carcinoma: a study on 23 cases.

A. Hauschild[1], M. Kohne[1], P. Rudolph[2], M. Tiemann[2], E. Christophers[1], M. Weichenthal[1]

[1]University of Kiel, Dpt. of Dermatology, 24105 Kiel, Deutschland
[2]University of Kiel, Dpt. of Hematopathology, 24105 Kiel, Deutschland

Merkel cell carcinoma (MCC) is a rare skin tumor of neuroendocrine origin, which is clinically characterized by a high risk of local recurrence and metastasis. As a potential prognostic factor, the proliferation activity of 23 merkel cell carcinomas from 5 males and 18 females with a median age of 75 years (range 60-89) was analyzed. A broad spectrum of monoclonal antibodies against the Ki-S2, Ki-S4, Ki-S11, CD-44, p16, p27, and p53 antigens has been evaluated immunohistochemically in this study. Primary tumors had a median diameter of 2.0 cm (range 0.5-6.5cm) and were located on the head and neck in 7 cases, and in 16 cases on the limbs. At the time of diagnosis, lymphnode involvement was present in two patients. In 21 patients the disease was confined to the primary tumor only. Most patients received wide local excision (³ 3cm side margins), in some cases additional loco-regional radiation therapy was applied. After a median follow-up of 69 months, progression had occurred in nine patients (39.1%), including those with metastasis at initial presentation, and four patients (17.4%) had died of the disease.Age at diagnosis was a major prognostic factor for disease free survival (DFS), while primary site and gender showed some differences, but did not reach statistical significance. Due to a relatively low tumour related mortality, prognostic factors for overall survival (OS) could not be elaborated. The primary treatment did not affect DFS or OS, although local recurrence was significantly correlated to initial safty margins of less than 3cm.In the semiquantitative analysis of immunohistochemical stainings, potential progression markers showed only little differences in DFS ($p > 0.05$). In contrast, the Ki-S11 activity appeared to be a significant progression marker in MCC patients with respect to DFS ($p<0.001$) .The recently described mouse monoclonal antibody Ki-S11 recognizes a fixation-resistant epitope of the Ki-67 protein and is devoid of cross-reactivities. The antibody stains all cycling cells, but not resting (G0) or terminally differentiated cells. The use as a reliable proliferation marker with prognostic significance has already been demonstrated in patients with squamous carcinoma, and may therefore be promising in MCC too.

P149

Mutations of the BRAF gene in melanocytic skin lesions of various dignity

A. S. Yazdi[1], G. Palmedo[2], U. Puchta[1], M. J. Flaig[1], H. Kutzner[2], C. A. Sander[1]

[1]Ludwig-Maximilians-Universität München, Dermatologie, 80337 München, Deutschland
[2]Dermatohistopathologisches Gemeinschaftslabor, 88048 Friedrichshafen, Deutschland

Genes of the RAF family mediate cellular responses to growth signals. A recent study reported about a T1796A mutation in exon 15 of the BRAF gene in a high frequency in melanoma cell lines and in 6 of 9 primary malignant melanomas (MM). The high frequency of BRAF mutations was considered to be a possible target of therapy of advanced stages of malignant melanoma. To clarify the role of BRAF mutations in tumorgenesis of malignant melanoma we screened BRAF mutations from malignant melanomas, MM in situ, various types of melanocytic nevi, Spitz nevi and MM with an underlying nevus. The frequency of the specific T1796A mutation in malignant melanoma was considerably low (28/97). Moreover we could detect the mutation in several non-malignant melanocytic skin lesions (37/175). Spitz nevi (0/63) and blue nevi (0/19) did not show any mutated cases, while papillomatous nevi showed the highest frequency of mutations (20/27).The combined lesions of a malignant melanoma in a nevus were either mutated both in the microdissected nevus (3/14) and the melanoma cells or both lesions were wild-type (10/14), only one case showed the mutated nucleotide solely in the MM part. We screened 300 melanocytic skin lesions for the presence of the specific BRAF mutation. As the mutation could be found in similar frequency in both malignant and benign lesions, other so far unknown cofactors must play a role in carcinogenesis.

P150

The Role of Transforming Growth Factor β (TGF-β) in Cutaneous Squamous Cell Carcinoma

F. Weber[1], G. M. Halliday[2]

[1]University of Innsbruck, Dept. of Dermatology and Venereology, 6020 Innsbruck, Oesterreich
[2]University of Sydney, Dept. of Dermatology, 2006 Sydney, Australia

Transforming growth factor β (TGF-β) is known to play an important role in tumorigenesis and tumour progression due to its effects on cell prolfieration, anti-tumour immunity and angiogenesis. To study the role of TGF-β in cutaneous squamous cell carcinoma (SCC) in vitro and in a mouse model in vivo we stably transfected a murine cutaneous SCC cell line with the murine gene for TGF-β_1.
In contrast to normal keratinocytes TGF-β_1 enhanced the cell proliferation rate of this malignant cell line. The transfected cells were injected subcutaneously into syngeneic C3H/HeN mice and athymic nude mice. In vivo the effect of TGF-β_1 on tumour progression was dependent on the level of cytokine expression after transfection: low level of TGF-β_1 inhibited whereas medium and high levels of TGF-β_1 increased tumour growth in both mice strains.
Immunohistochemistry of the tumours revealed increased T-cell and Dendritic cell (DC) density in the low TGF-β_1 expressing tumours and decreased infiltration in the medium and high TGF-β_1 expressing tumours.
TGF-β_1 production in the subcutaneous tumours caused a reduction of Langerhans cells in the epidermis above the tumours.In vitro adhesion of DCs on tumour cell layers and in vitro migration of DCs out of the tumour tissue was enhanced by low TGF-β_1 and reduced by medium and high TGF-β_1 production. Medium and high TGF-β_1 production of transfected tumour cells caused a significant reduction of Langerhans cells in the tumour-draining lymph nodes. Only low TGF-β_1 expression significantly enhanced tumour angiogenesis.
These results underline the role of TGF-β_1 in tumour progression and show that the effect of TGF-β_1 is dependent on the level of cytokine expression.

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Overexpression of $p16^{ink4a}$ in hpv positive dysplastic keratinocytes of non-melanoma skin cancer

I. Nindl[1], T. Meyer[2], C. Ulrich[1], T. Schmook[1], R. Arndt[2], W. Sterry[1], E. Stockfleth[1]

[1]Charité, Humboldt-University, Department of Dermatology, 10117 Berlin, Deutschland
[2]IPM-HH, Pathology and Molecularbiology, 22339 Hamburg, Deutschland

Background: The cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$ was identified as a specific biomarker in high-risk HPV infected cervical (pre)cancer lesions. We examined the overexpression of $p16^{INK4a}$ in normal, benign, and skin cancer lesions depending on the presence of HPV DNA.
Patients and Methods: Biopsies of 15 patients with normal histology (3), psoriasis (2), verrucae vulgares (2), actinic keratoses (1), Bowen carcinoma (1), morbus Bowen (3), and squamous cell carcinoma (3) were analysed. Forty-four biopsies of 15 patients were immunostained using the monoclonal antibody E6H4 specific for $p16^{INK4a}$. HPV status was assessed by a PCR system to detect so far all known HPV types. MY- (MY09/MY11 and MYN9/MYN10), CP- (CP65/CP70 and CP66/CP69) nested PCR, and 3 single PCR methods CN1, CN3, and CN4 were used in a first step and HPV typing was performed by RFLP analysis and subsequent hybridization of the PCR products. The quality of the specimens was controlled by β-globin DNA PCR and only β-globin positive patients were included in this study.
Results: HPV DNA was detected in all actinic keratoses (1/1), morbus Bowen (3/3), Bowen carcinoma (1/1), and squamous cell carcinoma (3/3), in 50% (1/2) of verrucae vulgares, in 66% (2/3) of normal skin and in none of psoriasis vulgaris. $P16^{INK4a}$ expression was not detected in normal keratinocytes, psoriasis vulgaris, and verrucae vulgares. Positive $p16^{INK4a}$ staining was detected in up to 80% of dysplastic keratinocytes of all actinic keratoses, Bowen carcinoma, morbus Bowen, and squamous cell carcinoma. HPV DNA was present in all $p16^{INK4a}$ positive specimens.
Conclusions: All HPV positive non-melanoma skin cancer were $p16^{INK4a}$ positive. $P16^{INK4a}$ staining may become a useful marker for non-melanoma skin cancer to detect dysplastic keratinocytes infected with HPV and can possible differentiate between high-risk and low-risk HPV infected skin cancer.

P152

Description of a novel antigen expressed in activated dermal cells and at the tumor-stroma-border of dermal tumors in situ.

U. Anderegg[1], M. Sticherling[2], U. Haustein[2], A. Saalbach[1]

[1]Sächsische Akademie der Wissenschaften zu Leipzig, 04103 Leipzig, Deutschland
[2]Universitätshautklinik Leipzig, Experimentelle Dermatologie, 04103 Leipzig, Deutschland

In previous investigations we isolated a novel cDNA that is induced in fibroblasts by supernatants from malignant melanoma cell lines in vitro. This cDNA, *Mel4b3*, is induced by TGFβ and Interleukin-1 in fibroblasts, keratinocytes and endothelial cells in vitro.
The mRNA was detected in situ in skin biopsies of malignant melanoma, sqamous cell carcinoma, but not in benign naevi or normal skin. The tumor associated expression was mostly found at the tumor-stroma border. Interestingly, often cells of the outer root sheath of the hair follicle expressed this mRNA also in healthy skin.Sequence comparison revealed high homology to KIAA1866, a model reference sequence that was predicted from NCBI contig NT-029991 by automated computational analysis.
However, the predicted protein coding sequence of KIAA1866 was distal from the open reading frame found in our sequencing work for *Mel4b3*. We developed monoclonal antibodies against peptide sequences of *Mel4b3* and screened the expression of the mRNA as well as of the detectable antigen in order to test whether this peptide co-localizes with the mRNA message in probes from normal skin and skin tumors, especially malignant melanoma.
Using in situ hybridization and immunohistochemistry we could show that the predicted peptide obviously exists and the immuno-histochemical data clearly co-localize with mRNA expression in the skin specimen investigated.
From these studies we conclude that the corresponding antigen might be associated with cell activation found in tumor development and hair growth.

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Caspase-independent induction of apoptosis by the proapoptotic Bcl-2-related protein Bcl-X_S in human melanoma cell lines

A. M. Hossini[1], J. Eberle[1], L. F. Fecker[1], C. E. Orfanos[1], C. C. Geilen[1]

[1]University Medical Center Benjamin Franklin, The Free University of Berlin, Department of Dermatology, 12200 Berlin, Deutschland

The Bcl-X gene gives rise to at least two alternative splice products with opposite functions. Whereas Bcl-X_L inhibits apoptosis through heterodimerization with proapoptotic members of the Bcl-2 protein family, Bcl-X_S antagonizes the antiapoptotic functions of Bcl-2 or Bcl-X_L and is therefore proapoptotic. In a previous study, we reported that Bcl-X_S overexpression is capable of inducing apoptosis in human melanoma cell lines, and we could further show that combination of Bcl-X_S overexpression with the proapoptotic stimuli Etoposide, DTIC, Ceramide and Fas agonistic antibody resulted in additive proapoptotic effects. In order to evaluate whether Bcl-X_S may be suitable for an anti-melanoma therapy, we investigated the pathway of Bcl-X_S-mediated apoptosis and the effect of Bcl-X_S overexpression on melanoma growth in vivo. In melanoma cells stably transfected with Bcl-X_S under control of a doxycycline-responsive promoter (SKM13-Bcl-X_S), typical marker proteins of apoptotic cascades were investigated by Western blotting. These investigations did not show higher levels of cytochrome c in the cytosol of Bcl-X_S transfected cells, however, cytochrome c was lost in mitochondria after induction of Bcl-X_S. Activation of caspase-8 and caspase-3 was only weak and transient, and neither cleavage of Bid (caspase-8 substrate) nor ICAD or PARP (caspase-3 substrates) was detectable. Consistently, we found mitochondrial release of apoptosis inducing factor (AIF) both 6 h and 48 h after Bcl-X_S-induction. For in-vivo experiments, SKM13-Bcl-X_S melanoma cells were injected subcutaneously into nude Balb-C mice. For promoter induction, one group of mice received doxycycline with the drinking water whereas the control group was left untreated. Average tumors size was significantly reduced in treated animals 7 weeks after induction of Bcl-X_S (5-times).These data indicate a caspase-independent induction of apoptosis by Bcl-X_S. Although cytochrome c release was not detected due to Bcl-X_S overexpression, the mitochondrial depletion of cytochrome c and release of other proapoptotic factors such as AIF seem to be characteristics of Bcl-X_S-induced apoptosis. Animal experiments may further suggest that Bcl-X_S overexpression may provide a novel proapoptoic tool against melanoma.

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Activation of MAGE-3 specific T helper cells from healthy donors and melanoma patients by peptide-loaded and Ii/MAGE fusion construct transfected dendritic cells.

M. Berg[1], B. Winzen[2], N. Koch[2], T. Bieber[1], S. Koch[1]

[1]Dept. of Dermatology, Lab. of Immunobiology, 53105 Bonn, Deutschland
[2]Inst. of Zoophysiology, 53117 Bonn, Deutschland

$CD4^+$ T lymphocyte responses are important for anti-tumor immune reactions as has been demonstrated in animal models. Characterization of the $CD4^+$ T helper cell epitope repertoire on melanoma antigens and specific targeting of endogenously synthesized antigens to the class II loading compartment would improve decisively the efficacy of peptide-based immunization protocols in neoplastic patients.
MAGE-$3_{171-185}$ peptide has been shown to bind promiscuously to MHC II molecules of all donors tested. Therefore, we used mature dendritic cells (DCs) as antigen presenting cells to stimulate MAGE-3 specific T cells of healthy donors and melanoma patients. Activation of the T cells was determined using an ELISPOT assay for gamma interferon and by measuring T cell proliferation. Using peptide-loaded mature DCs, we could stimulate T cells from all healthy donors tested and from 6 of 11 melanoma patients. MAGE-$3_{171-185}$ peptide specific T cells could be expanded by weekly restimulation. To improve the loading of MHC II molecules and thereby enhance efficacy of antigen presentation, we transfected immature DCs of a healthy donor with a fusion construct consisting of the MHC II associated invariant chain and MAGE-$3_{171-185}$ sequence by means of electroporation. 30 % of transfected DCs expressed the transgene, viability after transfection was at 60 %. Such transfected DCs were superior to peptide-loaded DCs in their capacity to stimulate primary MAGE-$3_{171-185}$ specific T cells, which produced more gamma interferon and interleukin 12. This novel antigen delivery system holds promise for a variety of clinical applications including cancer therapy.

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Genetic analysis of BRAF mutations in malignant melanoma metastases

N. Roskot[1], D. Koczan[2], S. Ibrahim[2], H. Thiesen[2], G. Gross[1], M. Kunz[1]

[1]Universität Rostock, Dermatologie und Venerologie, 18055 Rostock, Deutschland
[2]Universität Rostock, Institut für Immunologie, 18055 Rostock, Deutschland

The RAS-RAF-MEK-ERK signaling pathway is of central importance for cell cycle control and cell proliferation. Activating mutations in RAS and RAF genes have been identified in a variety of malignant tumors. Very recently it could be shown that BRAF mutations occur in more than 60% of primary malignant melanomas. Thus, the BRAF gene might play a central role in malignant melanoma pathogenesis. In the present report we analyzed mutations in this gene in well characterized melanoma cell lines of different aggressiveness and in primary melanomas and melanoma metastases. We addressed the question whether melanoma metastases display the same mutations found in primary melanomas, or, may even have acquired new mutations in the BRAF kinase domain, leading to enhanced BRAF activity. In order to screen large series of tumors we established denaturing high performance liquid chromatography (DHPLC) for the analysis of BRAF mutations. By use of direct sequencing and DHPLC analysis it could be demonstrated that low aggressive melanoma cell lines displayed the published mutations in the BRAF kinase domain, while two highly aggressive cell lines tested did not. One of the highly aggressive melanoma cell lines showed one additional mutation in the BRAF kinase domain. Taken together, the presented data provide evidence for an acquisition of further muta

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Effective DEAE dextran mediated transfection of primary blood lymphocytes, dendritic cells, and immature hematopoietic cells for use in functional DNA repair assays

K. Thoms[1], J. Baesecke[2], C. Neumann[1], S. Emmert[1]

[1]Universitaet Goettingen, Dermatologie, 37075 Goettingen, Deutschland
[2]Universitaet Goettingen, Haematologie und Onkologie, 37075 Goettingen, Deutschland

The host cell reactivation (HCR) assay measures the ability of transfected cells to repair plasmid DNA damage as reflected in the recovery of luciferase activity. We established the HCR assay to measure DNA repair capacity of primary blood lymphocytes, dendritic cells, and $CD34^+$-cells using an optimized DEAE dextran transfection protocol. $2x10^5$ cells were transfected with 250 ng plasmid DNA. Primary blood lymphocytes were isolated by Ficoll gradient and, after cryopreservation, PHA stimulated for 3 days. Immature dendritic cells were derived from primary blood monocytes (6 day cultures in the presence of IL-4 and GM-CSF). $CD34^+$-cells were isolated from cord blood by MACS and, after cryopreservation, cultured in stem cell medium. We obtained luciferase activities 1000-fold, 200-fold, and 350-fold above background activity with undamaged plasmids in the different cell types, respectively.
In addition, we established a plasmid shuttle vector mutagenesis assay using primary blood lymphocytes. $2x10^6$PHA stimulated lymphocytes were transfected with 250 ng reporter plasmid using DEAE dextran. After 2 days the plasmids were harvested from the cells and transformed into bacteria. Blue colonies indicate complete DNA damage repair whereas white bacterial colonies indicate mutations resulting from unrepaired DNA damage.
These two functional DNA repair assays established with different primary cells will enable us to test individuals for their capacity of different DNA repair pathways like nucleotideexcision, baseexcision, or double strand break repair depending on the type of DNA damage induced in the plasmid. Ultimately, the identification of differences in individual DNA repair capacities might lead to the identification of differences in individual cancer susceptibility.

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Intravenously-injected paclitaxel encapsulated in cationic liposomes diminishes tumor angiogenesis and melanoma growth in a 'humanized' SCID mouse model

R. Kunstfeld[1], G. Wickenhauser[1], U. Michaelis[2], M. Teifel[2], W. Umek[3], K. Naujoks[2], K. Wolff[1], P. Petzelbauer[1]

[1]University of Vienna, Department of Dermatology, Division of General Dermatology, 1090 Wien, Oesterreich
[2]mbt Munich Biotechnology AG, 82152 Martinsried, Deutschland
[3]University of Vienna Medical School, Department of Obstetrics and Gynecology, 1090 Wien, Oesterreich

Paclitaxel is an alkaloid which inhibits endothelial cell proliferation, motility and tube formation at nanomolar concentrations. Cationic liposome preparations have been shown to target blood vessels. We wished to explore the possibility that paclitaxel encapsulated in cationic liposomes carries paclitaxel to blood vessels and thereby provides an anti-angiogenic effect. We used a humanized SCID mouse melanoma model, which allows to analyze tumor growth and tumor angiogenesis in an orthotopic tumor model. Here, human melanoma cells grow on human dermis and are in part nourished by human vessels. We show that paclitaxel encapsulated in liposomes prevents melanoma growth and invasiveness and improves survival of mice. Moreover, liposome-encapsulated paclitaxel reduces vessel density at the interface between the tumor and the human dermis and reduces endothelial cell mitosis to background levels as seen in mouse liver endothelium. In contrast, equimolar concentrations of paclitaxel solubilized in Cremophor EL® had only insignificant effects on tumor growth and did not reduce the mitotic index of endothelium *in vivo*, although the anti-proliferative effect of solubilized paclitaxel in Cremophor EL® *in vitro* was identical to that seen with liposome-coupled paclitaxel. In conclusion, we present a model of how to exploit cytotoxic effects of compounds to prevent tumor growth by using cationic liposomes for targeting an anti-proliferative drug to blood vessels.

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Expression of MUC 1 and EpCAM in Merkel cell carcinomas: implications for immunotherapy

H. Kurzen[1], S. Kaul[2], U. Egner[1], M. Deichmann[1], W. Hartschuh[1]

[1]Universität Heidelberg, Hautklinik, 69115 Heidelberg, Deutschland
[2]Universität Heidelberg, Frauenklinik, 69115 Heidelberg, Deutschland

Merkel cells are neuroendocrine cells of the skin that share many features with simple epithelial cells, like the expression of low molecular weight cytokeratins. The glycoprotein Ep-CAM is a homophilic cell-cell adhesion molecule, present in most simple, pseudo-stratified and transitional epithelia and the tumors derived thereof. It shows strong expression in basal cell carcinomas while squamous cell carcinomas lack expression of this antigen. MUC 1 is a well established marker for squamous cell carinomas generally secreted by glandular epithelial cells. We compared the expression of Ep-CAM and MUC 1 in 30 cases of Merkel cell carcinomas and 12 metastases using a nickel-enhanced ABC technique. We found MUC 1 and Ep-CAM expressed in Merkel cells and in about 85% and 73% of all Merkel cell carcinomas irrespective of clinical outcome. In metastases both antigens were expressed in ~70% of the cases. Like in mammary carcinoma, we found no difference in MUC 1 expression between metastasizing and not metastasizing primaries. MUC 1 expression was not correlated to clinical outcome. Ep-CAM expression seems to be higher in primary Merkel cell carcinomas that metastasize than in those that did not lead to metastasis. However, due to the low number of primaries that did not metastasize (9), these figures have to be interpreted cautiously. In conclusion, Merkel cells and the majority of Merkel cell carcinomas express Ep-CAM and MUC 1. This opens the door for treatments based on monoclonal antibodies against these antigens, already established in other tumor entities. Also, vaccination therapies might be developed.

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Melanoma antigens expression of melanoma cell lines obtained from fine-needle-biopsies are representative for the in situ tumor

J. Kamarachev[1], K. Jungius[2], G. Burg[1], F. O. Nestle[1]

[1]University Hospital Zurich, Department of Dermatology, 8091 Zurich, Schweiz
[2]Institute for Diagnostic Radiology, Department of Radiology - University Hospital Zurich, 8091 Zurich, Schweiz

Definition of the tumor antigen profile during immunotherapy is essential for monitoring of the tumor response to a given vaccine. Fine needle biopsy provides a tool for dynamic evaluation of tumour antigen profiles during disease evolution or therapeutic interventions at different tissue sites. We were able to expand melanoma cell lines out of fine-needle-biopsy-material obtained from 2 subcutaneous and 3 lymph node metastases in 4 patients. To answer the question whether these cell lines are representative for the antigen profile of the in situ tumour we performed immunohistochemistry on histological sections of the initial in situ tumour and cytospin preparations of the cell lines. We compared the expression of the melanoma antigens MAGE 3, Melan A, Tyrosinase, GP 100 as well as HLA-class I of our melanoma cell lines with the expression of those antigens in the initial metastases. MAGE 3 was detected in all 5 biopsy-specimens and melanoma-cell-line cytospins. Melan A was strongly expressed in 2 biopsies and in the respective cell lines. In the remaining 3 biopsies it showed a heterogeneous, focal staining. Of the corresponding cell-culture cytospins one was also heterogeneously stained and 2 were negative for Melan A. Tyrosinase expression was detected in 3 tumour specimens and in all 3 of the corresponding cell lines. The two cell cultures corresponding to the tyrosinase-negative metastases were also negative. Class I expression was negative in 2 metastases and in the respective cell lines. Our data show that the antigen profile of melanoma cell lines, yielded from fine-needle biopsies is representative of the in vivo tumour and represents therefore a valuable tool for future trials in melanoma immunotherapy.

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Tumor-specific Th1 cells inhibit tumor angiogenesis in mice

H. Braumueller[1], B. Pichler[2], A. Sakrauski[1], M. Kneilling[1], K. Ghoreschi[1], M. Schwaiger[2], W. Weber[2], M. Roecken[1]

[1]Ludwig-Maximilians-Universitaet Muenchen, Klinik und Poliklinik fuer Dermatologie und Allergologie, 80337 Muenchen, Deutschland
[2]Technische Universitaet Muenchen, Nuklearmedizinische Klinik und Poliklinik, 81675 Muenchen, Deutschland

RIP1-Tag2 transgenic mice are an established model for multistage tumorigenesis and angiogenesis. In these mice the large T antigen (Tag) of the Simian Virus 40 is only expressed in insulin-producing beta cells of the pancreas, leading to the successive development of islet cell hyperplasia, adenomas, and finally invasive, highly vascularized carcinomas. Subsequently the mice die of hypoglycemia at an age of about 15 weeks. We investigated, whether adoptive transfer of Tag-specific Th1 cells alone can inhibit tumor growth and tumor angiogenesis.Th1 cells were generated by stimulating isolated $CD4^+$ cells transgenic C3H mice with T antigen and antigen presenting cells in the presence of CpG 1668 oligonucleotide. At an age of 6-7 weeks adoptive transfer of Th1 cells started and was performed weekly. We followed blood glucose levels and histology. To evaluate, whether the mechanism of this therapy involves anti-angiogenesis we used a glycosylated RGD-peptide with selective binding to the integrin alpha-v beta-3 as a marker for endothelial cell activation. Adoptive transfer of Th1 cells resulted in a doubling of the life span of RIP-Tag positive mice. Surprisingly, Th1 therapy did not induce diabetes even though all islet cells express Tag. At 12 weeks histology showed large, vascularized insulinomas in sham-treated mice. In sharp contrast, Th1-treated animals had only small poorly vascularized adenomas. Depletion of $CD8^+$ cells influenced tumor development neither in sham-treated nor in Th1-treated RIP1-Tag2 mice. Several studies showed that the integrin alpha-v beta-3 plays a critical role in the angiogenic process. In untreated mice Gluco-RGD uptake started to increase at 7 weeks of age and reached 8-fold higher levels at 8 weeks. In sharp contrast, over the same period Gluco-RGD uptake remained at back ground levels in Th1-treated mice.Our results show that adoptive transfer of tumor-specific Th1 cells is a powerful tool for antitumor treatment in mice. Treated mice did not get any sign of autoimmune diseases although tumor growth is suppressed. The mechanism of this highly efficient delay seems to be the inhibition of angiogenesis.

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Association of HPV infections with cutaneous tumors of immunosuppressed patients

T. Meyer[1], I. Nindl[2], R. Arndt[1], C. Ulrich[2], E. Christophers[3], W. Sterry[2], E. Stockfleth[2]

[1]IPM, Virology, 22339 Hamburg, Deutschland
[2]Charite, Humboldt University, Dermatology, 10117 Berlin, Deutschland
[3]University of Kiel, Dermatology, 24105 Kiel, Deutschland

Background: Skin cancer is the most common malignancy diagnosed in organ transplant recipients. Besides immunosuppression and UV-radiation HPV infection was also suggested to be involved in the development of these cancers. To analyze the possible predominance of particular HPV types in skin cancers we analyzed the prevalence of HPV types in different skin tumors.
Patients and Methods: Biopsies of different benign and malignant skin tumors (81 verrucae vulgares, 28 actinic keratoses, 36 Bowen‘s disease, 42 cutaneous SCC) as well as 63 normal skin samples from transplanted and non-transplanted patients were analyzed for the prevalence of HPV DNA by a PCR system designed to detect all HPV types known so far.
Results: HPV DNA was detected more frequently in SCC of transplant recipients (75%) compared with the same lesions of non-immunosuppressed patients (47%). Similar HPV prevalences were found in cutaneous warts (91% vs 94%), premalignant skin tumors (38% vs 36%), and normal skin specimens (17% vs 16%) of both patient populations. Overall, more than 40 different HPV types were identified. HPV types 5 and 8 were found more frequently in SCC (26%) than in precancerous (5%) or benign lesions (1%). All HPV 5- and HPV 8-positive SCC were from immunosuppressed patients.
Conclusions: Infection with HPV types 5 and 8 may represent an increased risk for SCC development in transplant recipients. These viruses should be considered preferentially in antiviral strategies aimed at the reduction of skin tumors in organ transplant recipients.

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Investigation of Psoriasin (S100A7) Expression in Human Skin Cancer

N. Soos[1], E. Wandel[2], P. Velasco[1], M. Weichenthal[1], U. Mrowietz[1], E. Christophers[1], J. Schröder[1], M. Sticherling[2], R. Gläser[1]

[1]University of Kiel, Department of Dermatology, 24105 Kiel, Deutschland
[2]University of Leipzig, Department of Clinical and Experimental Dermatology, 04103 Leipzig, Deutschland

The Calcium-binding S100-protein psoriasin was originally identified as an upregulated protein in psoriatic keratinocytes. Recently, elevated expression of this protein was found in *in situ* ductal breast carcinoma as well as in bladder squamous cell carcinoma. This study was initiated to investigate the role of psoriasin mRNA and protein expression in human epithelial skin tumors.

Skin samples were obtained from patients undergoing surgical tumor resection. Two punch biopsies were taken from each patient, one from the center of the tumor, the other one from non-involved skin. An optimized protocol for sample storage and RNA extraction was established to achieve maximum quality and yield of total RNA.
Realtime-RT-PCR with intron-spanning primer pairs revealed a higher relative transcription level for psoriasin mRNA in all tumor entities when compared to uninvolved skin (n=18). The median psoriasin mRNA expression was 5,6-fold higher in premalignancies (M. Bowen and *in situ* squamous cell carcinoma, n=5), 3,3-fold in basal cell carcinoma (n=10) and 4,4-fold in invasive squamous cell carcinoma (n=4).
To investigate the protein expression pattern of psoriasin, a newly available monoclonal antibody directed against recombinant psoriasin was used. The specificity of this antibody was confirmed by western blot analysis and blocking experiments with natural psoriasin isolated from healthy human heel stratum corneum. Psoriasin expression was found to be strongly correlated with the histopathological pattern of differentiation within the tumor samples.

Psoriasin may play a role in skin cancer and could serve as a diagnostic tool of particular tumor entities. Further investigations will address this aspect.

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Telomere-homologue oligonucleotides (T-oligos), all-trans retinoic acid (ATRA) and 1,25 dihydroxy-Vitamin D3 (VitD3) induce apoptosis in human melanoma cells

I. M. Hadshiew[1], K. Barre[1], A. Thies[2], I. Moll[1]

[1]University-Hospital Eppendorf, Dept. of Dermatology, D-20246 Hamburg
[2]University-Hospital Eppendorf, Institute for Neuroanatomy, D-20246 Hamburg

Single-stranded DNA-oligonucleotides, homologue to the 3´ overhang of human telomeres, have been shown to induce various DNA-damage responses, such as cell-cycle arrest, induction of DNA-repair genes and proteins, melanogenesis and apoptosis in normal human cells as well as Jurkat cells.We were able to demonstrate that three highly metastatic human melanoma cell lines (MV3, G361 and UISO-Mel6) undergo apoptosis after 24h hours of treatment with either 20µM T-oligo, 1µM ATRA or 10nM VitD3. As shown by TUNEL staining and subsequent FACS analysis and fluorescence microscopy, apoptosis was induced in all 3 cell lines; the T-oligo resulting in an increase in apoptosis between 50% and 70% compared to diluent treated controls, depending on cell line, while ATRA led to an induction of apoptosis of 20% to 46% and VitD3 18% to 48 %. In G361 cells, the strongest induction of apoptosis (69%) was seen after T-oligo treatment compared to 33% after ATRA treatment and 33% after VitD3 treatment. In MV3 cells, the induction was 57 % after T-oligo treatment compared to 18% after each of the 2 other treatments. For UISO-Mel6 cells the increase in induction was similar for all three agents (46% to 49%).While mechanisms by which ATRA and VitD3 regulate cellular differentiation and apoptosis are better understood, little is known for T-oligos. Nuclear receptors for both ATRA and VitD3 have been identified, however, no specific receptor or sensor has been identified for T-oligos. 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 action and cellular interactions for T-oligos. Potentially, combining ATRA, VitD3 and T-oligos with each other and with specific cytotoxic agents might enhance therapeutic options for malignant melanoma.

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Autocrine ephrin-B2 - Eph receptor tyrosine kinase signalling enhances integrin-mediated ECM-attachment and invasion of melanoma cells

S. Meyer[1], M. Guba[2], S. Flegel[2], B. Becker[1], E. Orso[3], M. Landthaler[1], T. Vogt[1]

[1]Universitaet Regensburg, Dermatologie, 93042 Regensburg, Deutschland
[2]Universitaet Regensburg, Chirurgie, 93042 Regensburg, Deutschland
[3]Universitaet Regensburg, Klinische Chemie, 93042 Regensburg, Deutschland

Eph receptors are a family of receptor tyrosine kinases (RTK) with a pivotal role in embryonic patterning and neuronal development. This is partially attributable to modulation of cell migration and ECM-adhesion by cell-cell signalling. Malignant melanomas (MM) overexpress certain ephrin ligands and the corresponding cell surface Eph-RTKs constitutively, suggesting autocrine stimulatory loops in MM. However, the functional consequences for MM progression are currently unknown. The aim of this study was to find out, whether ECM-attachment and invasion of MM cells are altered by ephrin-B2-EphRTK signalling. A panel of 20 cell lines from mouse and human melanoma and normal tissues was screened by RT-PCR for relative expression levels of ephrin-B2. In contrast to most other cancer cell lines, mouse B16 MM-cells lacked ephrin-B2 expression almost completely. A full-length mouse ephrin-B2 expression construct was stably transfected into B16-cells. Overexpression was confirmed by FACS and Western blotting. Attachment assays were performed by seeding transfectants onto surfaces coated with laminin (LN), fibronectin (FN), and collagen: Ephrin-B2 overexpressing clones showed a significantly increased attachment to LN and FN, but not to collagen, suggesting the involvement of specific integrins. Consistently, the increase of attachment could be completely blocked with specific integrin competitors like RGD peptide or anti-β1 monoclonal antibody. The acute initial FN-tethering of the melanoma cells was further stimulated by pre-incubating the cells with recombinant extracellular EphB4-receptor domain, simulating neighboring receptor expressing cells. The observed increase of attachment abilities correlated closely with an increased migratory competence of ephrin-B2 overexpressing cells in FN-coated Boyden chambers. Taken together, we collected evidence that autocrine bidirectional ephrin-B2-EphRTK signalling among MM-cells might be an important mechanism of activation of FN- and LN-binding integrins, which determine MM-cell adhesion, migratory competence, and possibly metastatic spread.

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Differential expression of matrix metalloproteinases in spontaneous and experimental melanoma metastases reflects the requirements for tumor formation

U. B. Hofmann[1], A. A. Eggert[1], K. Blass[1], E. B. Broecker[1], J. C. Becker[1]

[1]Universitaets-Hautklinik, 97080 Wuerzburg, Deutschland

Expression of matrix metalloproteinases (MMPs) and their activation in tumor cells as well as tumor surrounding stromal cells have been implicated in tumor cell invasion and metastasis. Here, we established a syngeneic model consisting of three murine melanoma cell lines which spontaneously form metastases to different degrees after subcutaneous injection (s.c.) into C57BL/6 mice. Subcutaneous tumors, lymph node metastases as well as spontaneous and experimental lung metastases allowed to characterize the differential expression of MMPs and TIMPs in relation to the microenvironment and the way of metastasis induction.

In vitro increased levels of secreted MMP-2 MMP-9 and TIMP-1 protein were detectable only in the most aggressive cell line B16G3.12BM2. Remarkably, only in this cell line active MMP-2 was present. TIMP-2 and MT1-MMP showed comparable levels in all cell lines. *In vivo* MMP-2, MMP-9 and MT1-MMP were predominantly expressed at the tumor-stroma border of subcutaneous tumors.

Remarkably, functional active MMP-2 was restricted to this tumor invasion front. In spontaneous lymph node and lung metastases a high number of tumor cells and tumor cell clusters expressed MMP-9 at the interior and periphery of the metastases, whereas these areas were predominantly negative for both MMP-2 and MT1-MMP. In contrast to spontaneous metastases tumor cell clusters of experimental lung did not express MMP-9.

These results indicate that expression of MMPs in melanoma metastases is not strongly influenced by localization but also the nature of metastases induction, suggesting that these different MMPs serve individual and specific roles during the different stages of metastasis formation.

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Tumor antigens in CTCL: mRNA Expression and sero-reactivity

S. Eichmüller[1], D. Usener[1], D. Thiel[1], D. Schadendorf[1]

[1]German Cancer Research Center (DKFZ), Skin Cancer Unit (D0900), 69120 Heidelberg, Deutschland

We have investigated the possible expression of tumor-specific targets in cutaneous T-cell lymphoma (CTCL) focusing mainly on so-called cancer-germline genes. cDNAs derived from 20 CTCL tissues and 4 CTCL cell lines were tested with 15 gene- and 4 gene family-specific primers by RT-PCR and confirmative Northern blotting. The most frequently detected mRNAs were LAGE-1 (55%, with only partial coexpression of the two splicing variants), cTAGE-1 (35%), MAGE-A9 (27%), and the GAGE-3to7 group (35%). Furthermore, we could detect NY-ESO-1 (21%) and a MAGE-A subgroup (15%), while sub-specification of the latter proved absence of MAGE-A2, -A3. SCP-1 was found in only one specimen and a several antigens could not been detected in any tumor tissue or cell line (MAGE-B, GAGE-1,2,8, and all four RAGE genes). 95% of all CTCL samples were positive for at least one of the frequent mRNAs in RT-PCR (LAGE-1, NY-ESO-1, cTAGE-1, MAGE-A9, and/or GAGE-3to7). Using a secondary SEREX approach we could detect sero-reactivity in sera of CTCL patients against recombinant GAGE-protein, MAGE-A1, -A3, -A6, and -A9, but not against LAGE-1b. We conclude, that certain cancer-germline genes can be detected frequently in CTCL and are able to elicit a systemic immune response. These candidate genes might therefore be promising targets for immunotherapeutic interventions in CTCL.

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Sero-reactivity of melanoma patients against MAGE-A and LAGE-1 proteins as analyzed by HYREX

D. Usener[1], A. Gerhardt[1], D. Schadendorf[1], S. Eichmüller[1]

[1]German Cancer Research Center (DKFZ), Skin Cancer Unit (D0900), 69120 Heidelberg, Deutschland

The humoral response to known cancer-testis antigens was investigated in melanoma patients using phage clones coding for 7 different MAGE-A / LAGE-1B proteins. These clones have been isolated using the newly developed HYREX (DNA hybridization analysis of recombinantly expressed cDNA libraries) approach. HYREX combines the advantage of a non-radioactive library screening method with the possibility of subsequently analyzing the serological response to the recombinant proteins. We isolated clones coding for MAGE-A1, -A3, ?A4b, ?A9 and ?A12, as well as LAGE-1B, and found between 13% and 27% sera (n=15) being reactive against individual tumor antigens. Additionally, a correlative expression analysis of MAGE-A1, -A3, pan-MAGE-A, and LAGE-1B was performed. We found the presence of specific antibodies was generally correlated with mRNA expression of the antigen within cell lines derived from the same patient with only two exceptions. While cross-reactivity of patients' IgG might play a role in these cases, antibodies from patients' sera were able to distinguish even the closely related MAGE-A3 and –A6. In general, the mRNA expression frequency was higher than the detected IgG responses. Antibody recognition of specific tumor antigens by patients' sera may be used for evaluating new antigens in their possible immunogenicity and serological tests could be used for monitoring purposes.

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Discrimination between gene expression patterns of the invasive margin and the tumour core of malignant melanomas

A. Roesch[1], B. Becker[1], W. Stolz[2], M. Dugas[3], M. Landthaler[1], T. Vogt[1]

[1]Universitaetsklinikum Regensburg, Klinik und Poliklinik für Dermatologie, 93053 Regensburg, Deutschland
[2]Krankenhaus München-Schwabing, Dermatologie und Allergologie, 80804 München, Deutschland
[3]Ludwig-Maximilians-Universitaet München, Medizinische Informatik, Biometrie und Epidemiologie (IBE), 81377 München, Deutschland

Genes that determine the invasive capacity of the invasive front of malignant melanomas (MM) have not yet been systematically investigated in vivo. Therefore, we combined laser pressure catapulting microdissection (LPC) with cDNA microarray technology (DermArray™ by Research Genetics, representing about 5700 genes) to systematically analyse differences in gene expression profiles between the invasive margin and the tumour centre in nine cases of vertical growth phase MM. Applying signal-to-noise statistical algorithms combined with hierarchical clustering, Phosphoenolpyruvate carboxykinase 1 (PEPCK), Homo sapiens similar to S. cervisiae SSM4 (TEB4), ribosomal protein L19, Homo sapiens similar to A. nidulans SudD suppressor of bimD6 homologue, Il-3 receptor α subunit, inositol 1,4,5-triphosphate 3-kinase isoenzyme and three anonymous ESTs were identified as class separating genes. These genes significantly discriminate between the invasive front and the tumour centre. Using this set of genes 15 out of 18 LPC-dissected MM-regions could be grouped correctly. We conclude that the candidate genes identified could spark further research on MM-progression and may provide novel prognostic parameters.

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Bisphosphonates induce apoptosis in melanoma cells

A. Forsea[1], C. Mueller[1], C. Riebeling[1,2], C. E. Orfanos[1], C. C. Geilen[1]

[1]University Medical Center Benjamin Franklin, Departement of Dermatology, 14195 Berlin, Deutschland
[2]Weizman Institute of Science, Departement of Biological Chemistry, 76100 Rehovot, Israel

At present, melanoma belongs to the most malignant tumours of the skin and mucous membranes due to its aggressive biological behaviour and tendency to generate early metastases. Malignant melanoma is characterized by its relatively high therapeutical resistance. Therefore, new therapeutical strategies need to be established. Bisphosphonates are used for the treatment of bone metastases and were initially thought to act via an inhibition of formation of osteoclasts from immature precursor cells or direct inhibition of resorption via induction of apoptosis in mature osteoclasts. Recently, evidence accumulated that bisphosphonates are potent inducers of apoptosis in several cancer cell types. These data indicate that the beneficial effect of bisphosphonates may result from a direct anti-tumour activity that may affect a broad range of metastasing tumours. The present study compared for the first time the in vitro effects of different bisphosphonates on melanoma cell lines. Three compounds with different mechanism of action and different antiresorptive potency were analyzed: a non-amino-bisphosphonate (clodronate) and two amino-bisphosphonates, pamidronate and zoledronate. It could be shown, that nitrogen-containing bisphosphonates inhibit cell proliferation, induce apoptosis and alter cell cycle progression. These effects were dose-and time- dependent, and were achieved at concentrations ranging from 10 to 100 µM. The pro-apoptotic effect was not inhibited by overexpression of Bcl-2 protein, suggesting that this class of bisphosphonates may stimulate a mitochondria-independent pathway for inducing apoptosis. In contrast, the non-amino-bisphosphonate clodronate had no effect on cell proliferation, apoptosis or cell cycle progression. These data indicate differences in the intracellular mechanism of action between the two types of bisphosphonates and suggest that nitrogen-containing bisphosphonates could be a promising novel therapeutic class for the treatment and/or prevention of melanoma metastases. Further studies are also necessary to elucidate the underlying mechanism of their pro-apoptotic effect in more detail and to establish the potential antitumoural role of different bisphosphonate derivatives in human melanoma.

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Characterization of carcinogen-induced skin tumors in C57BL/6 mice harboring the oncogenic R24C mutation in the cell cycle protein cdk4

J. Steitz[1], J. Lenz[1], S. Montag[1], G. Reinhard[1], S. Büchs[1], T. Wölfel[2], M. Malumbres[3], M. Barbacid[3], T. Tüting[1]

[1]Klinik für Dermatologie der Universität Bonn, 53105 Bonn, Deutschland
[2]3. Medizinische Klinik der Universität Mainz, 55131 Mainz, Deutschland
[3]Centro National de Investigaciones Oncologicas, 124 Madrid, Spanien

The oncogenic R24C mutation in the cyclin dependent kinase 4 (cdk4), an important cell cycle regulatory protein, has been identified in a melanoma cell line and was subsequently found in the germ-line of some melanoma-prone families. 129SV mice genetically engineered to carry a mutated cdk4(R24C) knock-in gene are susceptible to melanoma development after carcinogen treatment. The cdk4(R24C) knock-in has been introduced into C57BL/6 mice which are of particular interest for the evaluation of novel antigen-specific melanoma vaccines. Application of 25µg DMBA onto the skin of cdk4(R24C) knock-in C57BL/6 mice on day 4 after birth followed by either one UVB irradiation with 5,4 kJ/m2 on day 42 or by treatment with 5µg TPA twice weekly for 12 weeks induces skin tumors of melanocytic, epithelial, and mesenchymal origin. Tumor incidence at the age of 6 months is significantly increased when compared to wild-type C57BL/6 mice. We are currently characterising these skin tumors both macroscopically and microscopically. These studies provide the basis for the future evaluation of therapeutic strategies designed to enhance immune responses against melanoma and other skin tumors.

P171

Functional expression of CCR3 but not of CCR7 in lymphogenic melanoma metastases

A. Tun Kyi[1], F. Nestle[1]

[1]Universitätsspital Zürich, Dermatologische Klinik, 8031 Zürich, Schweiz

Involvement of chemokines and their cognate receptors in tumor metastasis has been shown recently. In this study, we analyzed melanoma cells for the expression of CCR3. We examined lymph node metastases derived (n=4) and CNS metastases derived melanoma cell lines (n=4) as well as normal melanocytes (n=3). Quantitative CCR3 expression at the mRNA and protein level as well as eotaxin internalisation could be detected in all the samples examined. Immunohistochemistry showed in vivo CCR3 expression in lymph node and CNS derived melanoma metastases as well as in melanocytes in normal skin. However, CCR3 mediated chemotaxis and actin polymerisation could only be observed in lymph node derived melanoma cell lines. CCR3 was not able to mediate chemotaxis in CNS derived melanoma cell lines and in normal melanocytes. We next looked at CCR7 expression which has recently been suggested to be involved in lymphatic metastasis. CCR7 expression was detected at the mRNA level but not on the protein level. Inhibition of G proteins and p38 abolished CCR3 mediated cell migration towards its ligand eotaxin in lymph node derived tumor cells. p38 phosphorylation could be confirmed by immunoblot analysis. To assess levels of CCR3 ligands eotaxin and RANTES, quantitative PCR was performed. mRNA amounts of these chemokines were highest in lymph nodes. Taken together, our data suggests that restored CCR3 signaling in melanoma cells leads to CCR3 mediated cell migration involving the p38 signaling pathway. This may result in metastasis to lymph nodes which express peak levels of the respective ligands.

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L1 adhesion molecule in the progression of malignant melanoma.

J. Reichrath[1], A. Abu-Dahi[1], M. Fogel[2], S. Mechtersheimer[3], T. Georg[4], A. Smirnov[2], M. Husar[2], W. Tilgen[1], P. Altevogt[3], P. Gutwein[3]

[1]Universität des Saarlandes, Hautklinik und Poliklinik, D-66421 Homburg
[2]Kaplan Hospital, Dept. of. Pathology, 33245 Rehovot, Israel
[3]Deutsches Krebsforschungszentrum, Tumorimmunologie, D-69120 Heidelberg
[4]Institut für Med. Biometrie, Epidemiologie und Informatik, Universität des Saarlandes, 66421 Homburg, Deutschland

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

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Frequent Genetic Alterations of Ras Signaling Pathway Genes in Sporadic Malignant Melanomas

J. Reifenberger[1], C. B. Knobbe[2], K. W. Schulte[1], T. Ruzicka[1], G. Reifenberger[2]

[1]Heinrich-Heine-University, Department of Dermatology, D-40225 Duesseldorf
[2]Heinrich-Heine-University, Department of Neuropathology, 40225 Duesseldorf, Deutschland

The Ras signaling pathway is an important intracellular mediator of mitogenic stimuli in response to the activation of cell membrane associated growth factor receptors. Oncogenic mutations in Ras genes are frequent in various human cancers. Here, we have investigated 37 sporadic malignant melanomas (15 primary cutaneous melanomas and 22 melanoma metastases) and 5 melanoma cell lines for mutation and mRNA expression of the Ras genes *NRAS* (1p13.2), *KRAS* (12p12.1) and *HRAS* (11p15.5). All tumors were additionally analyzed for genetic alteration and aberrant expression of the *BRAF* gene (7q34), which encodes a Ras-regulated serine/threonine kinase. Mutational analysis using single strand conformation polymorphism (SSCP) analysis and DNA sequencing identified somatic *NRAS* mutations in 6 tumors (2 primary melanomas and 4 melanoma metastases) and 1 cell line (MEL-JUSO). One melanoma metastasis showed a somatic missense *KRAS* mutation. *HRAS* mutations were not detected. Eight primary melanomas, 6 melanoma metastases and 4 cell lines (SK-MEL-1, SK-MEL-3, COLO-849 and IGR-37) carried activating *BRAF* mutations affecting the hot-spot codon 599 in all instances. Investigation for *BRAF* amplification and/or mRNA overexpression did not reveal any aberrations. None of the tumors or cell lines with *BRAF* mutation demonstrated *NRAS* or *KRAS* mutations and *vice versa*. Thus, a total of 21 of 37 melanomas (57%), including 10 of 15 primary tumors (66%), carried mutations in one of the 4 investigated Ras signaling pathway genes. In line with the recent study by Davies *et al.* (Nature 2002; 417:949-54), our data therefore indicate that aberrant Ras signaling through mutation of either *NRAS*, *KRAS* or *BRAF* is of paramount importance in the molecular pathogenesis of sporadic melanomas.

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Mesenchymal-Amoeboid Transition in Melanoma Cells After Blocking of β1 Integrins

N. Daryab[1], J. Moeller[1], J. A. Eble[2], E. Bröcker[1], P. Friedl[1]

[1]University of Würzburg, Department of Dermatology, D-97080 Würzburg
[2]University of Münster, Institute of Physiological Chemistry and Pathobiochemistry, 48149 Münster, Deutschland

Tumor cell migration through connective tissue is mediated by adhesive cell matrix interactions provided by integrins. Therfore, integrins may serve as candidate anti-invasion target receptors. We have investigated, whether tumor cell migration in 3D ECM environments is fully abrogated by blocking of integrin function or whether compensation mechanisms exist to maintain migration. Using highly invasive and metastatic MV3 melanoma cells, β1 integrin function was reduced or antagonized by i) flow cytometric sorting for subsets expressing low integrin levels, ii) blocking anti-β1 mAb 4B4 at different concentration, and iii) rhodocetin, a selective α2β1 disintegrin. For migration in 3D collagen matrices, MV3 melanoma cells exclusively utilized α2β1 integrins for elongation, adhesion to collagen fibers, fiber bundling, force generation, and migration. Reduction of β1 integrin-mediated adhesion resulted in strongly reduced overall migration rates, however a significant level of residual migration was observed. At partial reduction of integrin-mediated adhesion by cell sorting or by subtotal adhesion blockade using mAb 4B4 or rhodocetin, the conversion from fibroblast-like spindle-shaped morphology to more spherical amoeboid morphodynamics was obtained at undiminished migration velocity (0,3 - 0.4 m/min). At total abrogation of integrin-mediated adhesion, residual migration at velocities from 0.03 0,1 m/min were sustained by slow amoeboid movement and propulsive squeezing provided by a dynamic and adaptive cytoskeleton. In conclusion, cell migration as a complex supramolecular process can be driven by a viscoelastic cytoskeleton after blocking of primary adhesion receptors, supporting residual tumor cell migration in 3D ECM environments by low- or non-adhesive mechanisms. Mesenchymal-amoeboid transition after abrogation of integrin function my hence represent a putative escape strategy upon pharmacotherapeutic anti-integrin therapy.

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Visualization of focalized proteolysis upon tumor cell migration through three-dimensional extracellular matrix

K. Wolf[1], E. Deryugina[2], A. Strongin[2], E. B. Broecker[1], P. Friedl[1]

[1]Universität Würzburg, Department of Dermatology,, D-97080 Würzburg
[2]The Burnham Institute, The Burnham Institute, 92037 La Jolla, CA, USA

Tumor cell invasion and migration through 3D extracellular matrix result from adhesive and proteolytic cell-matrix interactions. However, it is not clear how the cleavage of ECM fibers for removal of extracellular matrix barriers is performed without weakening adhesive and migratory force generation. We have developed a combined physical and biochemical approach to visualize and quantify the remodeling and focal degradation of collagen fibers. The migration of highly invasive and proteolytic HT-1080 cells expressing MT1-MMP as well as other proteases were investigated within a 3D fibrillar type I collagen matrix model. Using 4D-confocal backscatter imaging for physical detection of collagen fibers, the traction and bundling of collagen fibers generated by leading pseudopods was accompanied by focal coclustering of b1 integrins and MT1-MMP at these binding sites. At the cell trailing edge, migration was associated with the remodeling of collagen fibers and the generation of tube-like matrix defects, indicating structural matrix degradation upon cell movement. The subcellular location of collagenolytic activity was analyzed for migrating cells within collagen lattices containing quenched FITC-collagen, generating cell surface-associated spot-like focal fluorescence at locations of structural fibril cleavage. At the leading edge, focal fluorescence was localized at lateral portions of growing pseudopods and at locations of circular fiber insertions and cell constriction. At detachment sites, prominent collagenolytic activity was observed upon focal release of bundled and reorganized collagen fibers. Net collagenolytic activity was quantitatively examined by FITC-release upon migration within a FITC-labeled lattice. The specificity of the above detection techniques was shown by blocking of total enzymatic activity using a cocktail of broad-spectrum protease inhibitors which lead to the abrogation of structural fiber degradation and remodeling, FITC release, as well as focal fluorescence generation at fiber insertions. In conclusion, direct detection of tightly controlled focal degradation of collagen fibrils at both laeding and trailing edge of live cells is possible, showing the contribution of pericellular matrix remodeling upon tumor invasi

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Fibroblast Activation Protein (FAP): Evaluation of Tet-inducible FAP in melanoma cell lines

R. D. Schubert[1], N. Kraut[2], J. Park[3], W. J. Rettig[2], R. U. Peter[4], P. Garin-Chesa[3,5], M. A. Huber[1]

[1]Universitätsklinikum Ulm, Universitätsklinik und Poliklinik für Dermatologie und Allergologie, 89081 Ulm, Deutschland
[2]Boehringer Ingelheim Austria GmbH, Onkologische Forschung, A-1120 Wien
[3]Boehringer Ingelheim Pharma KG, Onkologische Forschung, 88397 Biberach an der Riss, Deutschland
[4]Bundeswehrkrankenhaus Ulm, Dermatologie, 89081 Ulm, Deutschland
[5]Allgemeines Krankenhaus der Universität Wien, Klinische Pathologie, A-1090 Wien

The induction of Fibroblast Activation Protein (FAP) is a highly specific trait of activated tumor stroma fibroblasts in melanocytic skin tumors (Huber et al., *in press*). Whereas 30% of the nevi revealed additional FAP expression on subsets of melanocytic cells, melanoma cells from primary and metastatic melanomas were FAP negative. The downregulation of FAP on melanoma cells is currently not understood. In the present study we developed a functional *in vitro*-system for investigation of a possible role of FAP during melanoma carcinogenesis. FAP-inducible expression vectors were generated using the Tet-on-system (Gossen and Bujard, 1992) and stably integrated in FAP-negative melanoma cell lines. Using a chimeric transactivator, the transcription of FAP can be activated by tetracyclin via Tet-responsive elements (TREs). The Tet-controlled transactivator (rTA) represents a fusion of the wildtype (wt) Tet-repressor-protein (tetR) with the VP16-transactivation-domain of the herpes simplex virus. By modification of TetR to reverse TetR, the Tet-Response-Element in the target promotor region (of the FAP gene) can be bound in the presence of tetracyclin, and subsequently, the transcription of the target gene (FAP) is induced. Wt FAP and mutant FAP (which lacks catalytic activity by mutation of serine 624 to alanine) were cloned in the pTRE-vector. Subsequently, the regulator-plasmids pTet-on and pTet-off, and the "response-plasmids" (pTRE-wtFAP and pTRE-mutFAP) were stably integrated in FAP-negative melanoma cell lines (SK-Mel28, HT144, C32). Immunofluorescence and immunohistochemistry (using the FAP-specific monoclonal antibody F11.24) were applied to demonstrate that FAP is induced in these cell lines upon addition of doxycycline.In summary, the generation of inducible FAP in melanoma cell lines represents a highly relevant tool for further investigation of a possible role of FAP in the regulation of growth and transformation programs of melanocytic cells.

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Evidence that the induction of growth arrest in malignant melanoma by peroxisome proliferator-activated receptor (PPAR) γ agonists is partly independent of activation of PPARγ

R. Mößner[1], U. Krüger[1], U. Schulz[1], P. Middel[2], S. Schinner[3], L. Füzesi[2], C. Neumann[1], K. Reich[1]

[1]Georg-August University Göttingen, Department of Dermatology, 37075 Göttingen, Deutschland
[2]Georg-August University Göttingen, Dept. of Pathology, D-37075 Göttingen
[3]Georg-August University Göttingen, Dept. of Molecular Pharmacology, 37075 Göttingen, Deutschland

Peroxisome proliferator-activated receptor (PPAR)γ is a member of the nuclear receptor superfamily and believed to act as a heterodimer with the retinoid X receptor (RXR). Recently, we demonstrated expression of PPARγ in benign melanocytic naevi, primary cutaneous melanomas, melanoma metastases as well as in melanoma cell lines. We also showed that different PPARγ-specific agonists including the thiazolidindione derivates troglitazone and rosiglitazone dose-dependently inhibited cell proliferation in melanoma cell lines. The induction of growth arrest appeared to be through withdrawal from the cell cycle, but was independent from apoptosis. In support of this finding, troglitazone was found to downregulate the expression of cyclin D1 associated with a reduction of detectable hyperphosphorylated retinoblastoma (Rb) gene product. Several experiments were performed with the aim to further dissect the mechanisms involved in the growth inhibition induced by PPARγ agonists. First, we found that the sensitivity of melanoma cell lines towards the anti-proliferative effects of troglitazone correlated with the protein expression levels of PPARγ. Upon stimulation of cell lines with PPARγ agonists, PPARγ translocated from the cytoplasm to the nucleus as monitored by electron microscopy and immunogold labeling. However, pre-incubation of cell lines with the PPARγ-specific antagonist GW9662 did only reverse growth inhibition by approximately 50%. In addition, co-stimulation of melanoma cell lines with PPARγ and RXR agonists did not further enhance the effects of PPARγ agonists. We conclude that PPARγ agonists may inhibit cell growth in malignant melanoma at least partly independent of the activation of PPARγ.

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A new highly sensitive ELISA detects melanoma-associated serum fibronectin

M. Hofmann[1], P. Siegel[1], A. Lukowsky[1], W. Sterry[1], U. Trefzer[1]

[1]Humboldt-Universität, Hautklinik, 10117 Berlin, Deutschland

The monoclonal antibody SM5-1 is more sensitive than HMB-45, anti-tyrosinase or anti-MelanA/Mart-1 antibodies and is more specific than anti-S-100 in immunohistochemistry of melanoma lesions. The antigen recognized by SM5-1 could be identified as a melanoma-associated fibronectin variant (FNv). Both FACS as well as immunoelectron-microscopy demonstrate binding of SM5-1 both intracellularly and on the cell surface, suggesting that the FN variant might be secreted by melanoma cells. We therefore asked the question, whether the FNv could be detected in the serum of melanoma patients by means of an ELISA. Various combinations of mono- and polyclonal FN antibodies were used as capture antibodies in conjunction with peroxidase-linked (POD) SM5-1 as detection antibody. Furthermore, numerous variations of variables such as incubation times, reactions temperatures, reagents or different ELISA plates were evaluated so far. The best results were obtained when the commercially available mAb FN15 at a concentration of 5 µg/ml was used as a capture antibody o.n. at 40 ºC with NUNC plates. Blocking was done for 1 h at RT with 1% BSA and detection with 1 µg/ml SM5-1-POD for 90 min at RT. TMB was used as substrate for 10 min and the results measured at 450 nm. The 95-percentile was determined using the sera of 72 healthy, non-melanoma individuals at an OD of 0.05. A large number of 245 sera of melanoma patients in stage IV was then measured using this ELSIA, where we found a sensitivity of 76% (187/245). Remarkably, the two commercially available melanoma ELISAs, S-100 and MIA have a reported sensitivity which is also in this range. Therefore, we evaluated a total of 276 stage IV sera (including the 245 sera used in the SM5-1 ELISA) using the MIA-ELISA. The sensitivity here was slightly lower at 72% (199/276). Taken together, melanoma-associated fibronectin can be detected using this new sensitive ELISA. The sensitivity might be further increased by modifications, but already reaches the one of commercially available ELISAs.

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Unterschiedliche Frequenzen chromosomaler Translokationen in den Subtypen primaer kutaner B-Zell Lymphome

C. Hallermann[1], K. Kaune[1], C. Neumann[1], B. Gunawan[2], M. Vermeer[3], C. Tensen[3], R. Willemze[3], R. Siebert[4]

[1]Universität Göttingen, Dermatologie, 37075 Goettingen, Deutschland
[2]Universität Goettingen, Pathologie, 37075 Goettingen, Deutschland
[3]Universität Leiden, Dermatologie, 2300 Leiden, Niederlande
[4]Universität Kiel, Humangenetik, 24105 Kiel, Deutschland

Der Nachweis chromosomaler Translokationen bei extrakutanen B-Zell Lymphomen hat zur Charakterisierung von Lymphomentitaeten und zur Identifikation relevanter Onkogene gefuehrt. Als Konsequenz wurden zytogenetische Daten Bestandteil moderner Lymphomklassifikationen. Haeufig betreffen Translokationen bei B-Zell Lymphomen das IGH-Gen. Zu den haefigsten Translokationpartnern des IGH Lokus gehoehren bei follikulaeren Lymphomen und bei diffus grosszelligen B-Zell Lymphomen die Gene BCL2, CMYC und BCL6. Charakteristisch fuer das Marginalzonenlymphom hingegen ist eine Translokation des MLT-Gens. Unklar ist zur Zeit die Bedeutung dieser Translokationen fuer die Genese primaer kutaner B-Zell Lymphome(PCBZL). Methode: Mittels Interphase-FISH wurden bei 29 PCBZL das Auftreten von Translokationen der Genloki IGH, CMYC, BCL6, MLT untersucht (6 primaer kutane Keimzentrumszell Lymphome (PCFCCL), 14 grosszellige B-Zell Lymphome des Beins (LBCLL), 9 primaer kutane Marginalzonenlymphome (PCMZL)). Ergebnisse: Translokationen der untersuchten Loki liessen sich in 11/14 (79%) LBCLL jedoch in keinem PCFCCL oder PCMZL nachweisen. Translokationen des IGH-Lokus fanden sich bei 7/14 LBCLL. In 5 dieser Faelle konnte CMYC, in 2 Faellen BCL6 als Translokationspartner identifiziert werden. Darueber hinaus wiesen weitere 3 LBCLL eine Translokation des BCL6 Lokus auf, wobei der Translokationspartner in 2 Faellen unbekannt blieb, in einem Fall wurde der IG-λ Lokus als Partner identifiziert. Keines der 29 primaer kutanen B-Zell Lymphome hatte eine Translokation des MLT Lokus. In jedem der Faelle wurde eine IGH/BCL2 Translokation ausgeschlossen. Schlussfolgerung:Translokationen, welche in die Genese extrakutaner B-Zell Lymphome involviert sind, lassen sich in hoher Frequenz bei LBCLL, nicht jedoch bei den anderen Typen primaer kutaner B-Zell Lymphome nachweisen. Inwieweit dieses das klinisch aggressivere Verhalten der LBCLL erklaert muessen weitere Untersuchungen zeigen. Unsere Analyse zeigt, dass Translokationen des MLT-Gens und die Translokation t(14;18) nicht in die Genese kutaner B-Zell Lymphome involviert sind.

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Basic FGF and IL-1 alpha mediate the induction of proMMP-1 synthesis in fibroblasts co-cultured with high invasive BLM melanoma cells in vitro.

S. Löffek[1], P. Zigrino[1], G. von Muijen[2], C. Mauch[1]

[1]University of Cologne, Dept. of Dermatology, 50931 Cologne, Deutschland
[2]University Hospital Nijmegen, Dept. of Pathology, NL-6500HB Nijmegen

Tumour invasion and metastasis of malignant melanoma have been shown to require proteolytic degradation of the extracellular enviroment achieved primarily by enzymes of the matrix metalloproteinases (MMP) family. We have previously shown, that increased enzyme activity is localised at the border of tumour cells and the adjacent peritumoral connective tissue emphasising the crucial role of tumour-stroma interactions in the regulation of MMP activity.To address the question whether direct cell-cell contacts of melanoma cells and stromal fibroblasts or soluble factors, e.g. cytokines secreted by these cells might be involved in the regulation of MMPs, we co-cultured high invasive BLM melanoma cells and human dermal fibroblasts. Direct cell-cell contact induced proMMP-1 synthesis in skin fibroblasts. Moreover, treatment of fibroblasts with medium conditioned by BLM melanoma cells resulted in a strong induction of proMMP-1 synthesis. These results suggest that diffusible factors e.g. cytokines mediate this induction. Different soluble factors secreted by melanoma cells, e.g. IL-1, IL-6 and bFGF are known to be potent inducers of proMMP-1 synthesis.To address which cytokines are responsible for this effect, we performed competition assays with fibroblasts cultured in the presence of melanoma cell conditioned medium. Both, recombinant human IL-1 receptor antagonist and neutralising anti-IL-1 alpha antibodies dose dependently reduced but failed to abolish the synthesis of proMMP-1. Similarily, bFGF neutralising antibodies displayed a dose-dependent inhibition of proMMP-1 synthesis suggesting that induction of proMMP-1 synthesis observed in fibroblasts cultured in the presence of melanoma cell conditioned medium may be the resultant of the activity of multiple soluble factors as IL-1 alpha and bFGF. Interestingly, immunohistochemical studies on melanoma tissue increased IL-1 alpha proteins in those melanoma cells which are located in close proximity to the surrounding stroma. These data present evidence that inhibitors of cytokine-mediated activation of stromal fibroblasts by tumour cells could become important therapeutical drugs to inhibit tumour cell invasion.

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Differential Activity of Matrix Metalloproteinases and Cysteine Proteases in Melanoma Cell Lines Cultured in an *In Vitro* Invasion System

A. Klose[1], R. Dennhöfer[1], A. Willbrand-Hennes[1], G. van Muijen[2], T. Krieg[1], C. Mauch[1], N. Hunzelmann[1]

[1]University of cologne, Dept. Dermatology, 50931 Cologne, Deutschland
[2]University Hospital Nijmegen, Dept. Pathology, 6500HB Nijmegen, The Netherlands

Degradation of extracellular matrices and penetration of basement membranes (BM) are key events in tumor invasion and metastasis. Various classes of proteases and protease-inhibitors play a pivotal role in this dynamic process, and are regulated by manifold cell-cell and cell-matrix interactions. Assessment of these interactive mechanisms of tumor invasion requires an *in vitro* model system closely reflecting the complexity of the *in vivo* situation.We have recently described an optimized *in vitro* invasion system for melanoma cell lines, using dead deepidermized dermis (DDD) as an appropriate model to analyze tumor cell migration, stroma degradation and the underlying changes in expression levels of matrix degrading proteases. We have now investigated 4 melanoma cell lines which differ in their invasive behavior (low-, intermediate- or high-invasive) and seeded them either on the dermal side of the DDD or on the BM. Subsequently, these composites were analyzed with regard to cell invasion and in particular BM penetration. To analyze the responsible protease activities we performed *in situ* gelatin zymography of cryosections under two different buffer conditions: at neutral or at acidic pH values favoring either matrix metalloproteinases (MMPs) or cysteine proteinases respectively. Gelatinolysis in this system generally correlated with the invasiveness of the cells. Under neutral conditions gelatinolysis was detected in melanoma cells seeded on the dermal side but not when cultured on the BM. In contrast, at acidic pH, when cysteine proteases are preferentially active, proteolytic activity was detected in melanoma cells seeded on the dermis as well as in those seeded on the basement membrane side suggesting that the activity of cysteine proteases is important for the early invasion process. This increased proteolysis was parallel by increased amounts of lysosomal cysteine proteinase cathepsin B in the supernatants of these cells.The tumor invasion system herein described helps to improve our understanding of the complex mechanisms of matrix degradation and metastasis and revealed that cysteine proteases may crucially contribute to the invasion process.

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Antibodies in the serum of patients with melanoma-associated retinopathy bind to antigens expressed from different melanoma cell lines

C. Pföhler[1], G. Ladewig[1], W. Tilgen[1]

[1]The Saarland University Hospital, Department of Dermatology, 66421 Homburg/Saar, Deutschland

Melanoma-associated retinopathy (MAR) is a paraneoplastic syndrome in patients with metastatic melanoma. The pathomechanism is supposed to result from antibody production against not yet identified melanoma antigens; these antibodies cross-react with epitopes of retinal cells. Recently, immunofluorescent examinations were able to prove that serum from patients with subclinical MAR-suspicious symptoms contains antibodies that were able to react with retinal tissue from healthy donors as well as with autologous tumor tissue. In this study we screened serum samples from melanoma patients in the various stages of disease on cytospin sections of six different melanoma cell lines (BU-HOM, Mel Ju, Mel Juso, Colo 38, Sk Mel 37 and A375). Fibroblast were used as a negative control. All serum samples were used in a dilution of 1:200, detection was performed using a FITC-conjugated goat anti-human antibody. All serum samples showed antibody activity in three of six cell lines (BU-HOM, Mel Ju, Colo 38). In most cases the signals seemed to be located at the cell membrane because melanoma cells showed a ring-like staining pattern. Fibroblasts were always negative. We could show that serum from subclinical MAR-suspicious patients contains antibodies against not yet identified melanoma antigens which seem to expressed from several melanoma cell lines. Further investigations are required to identify and characterize the antigen(s) able to induce the production of antibodies cross-reactive with retinal tissue.

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The vitamin D system in malignant melanoma (MM), basal (BCC) and squamous cell carcinomas (SCC).

M. Seifert[1], M. Rind[1], L. Rafi[1], V. Meineke[2], T. Mitschele[1], W. Tilgen[1], J. Reichrath[1]

[1]Universitätskliniken des Saarlandes, Dept. of Dermatology, D-66421 Homburg
[2]Institute of Radiobiology, Federal Armed Forces Medical Academy, 80937 München, Deutschland

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

P184

Tissue-distribution of mast cells in cutaneous lymphomas

T. C. Fischer[1], N. Maaß[1], F. Serowka[1], P. Welker[1], D. A. Groneberg[2]

[1]Charite, Institute of Anatomy, 10115 Berlin, Deutschland
[2]Charite, Department of Pediatric Pneumology and Immunology, 13344 Berlin, Deutschland

Mast cells are likely to play a role in tumour cell-immune system interactions. As elevated numbers of tryptase-positive mast cells have previously been reported for bone marrow infiltrates of different lymphomas, the present study was designed to assess mast cell numbers and distribution in cutaneous lymphomas.
Skin biopsies of patients with cutaneous B-cell-, pleomorphic T-cell lymphomas and mycosis fungoides were subjected to immunohistochemistry for mast cell-specific tryptase and compared to normal control tissues.
In section of all three lymphoma types, elevated numbers of mast cells were found in the marginal zones of the tumour infiltrates when compared to control tissues. In the B-cell and pleomorphic T-cell lymphoma infiltrates, decreased numbers of tryptase-positive cells were present whereas in mycosis fungoides infiltrates, the numbers were increased. In peritumoural areas of pleomorphic lymphoma biopsies, the mast cell numbers were decreased if compared to the other lymphoma types or normal skin. Analysis of staining pattern revealed an activated mast cells status in the infiltrates if compared to the marginal zone.
In summary, the present studies demonstrate an altered mast cell tissue distribution and tryptase-staining pattern in different cutaneous lymphoma types. These changes may reflect a role of mast cells in tumour-immune system interactions.

P185

Members of the OATP family mediate active influx transport of xenobiotics and endogenous substances in normal human epidermal keratinocytes

J. M. Baron[1], R. Schiffer[1], F. Rodríguez[1], M. Neis[1], A. Dreuw[2], S. Joussen[1], H. F. Merk[1], F. Jugert[1]

[1]RWTH Aachen, Hautklinik, 52074 Aachen, Deutschland
[2]RWTH Aachen, Institut für Biochemie, 52074 Aachen, Deutschland

Normal human epidermal keratinocytes (NHEK) have been shown to express a cell type specific pattern of extrahepatic cytochrome P450 enzymes and efflux transport proteins showing that these cells metabolize and excrete a variety of xenobiotics. Recently transport proteins involved in the uptake of xenobiotics have been detected and here we analysed the mRNA- and protein-expression profiles and functional activities of these proteins in human keratinocytes in comparison to primary liver cells. The transporters studied included the subtypes A, B, C, D and E of the organic anion transporting polypeptide (OATP) family which are responsible for the uptake of various anionic and neutral molecules and especially organic cations - including drugs. Constitutive expression of OATP -B, -D and -E was shown for the first time in NHEK on a molecular level using RT-PCR and northern blot analysis, as well as in human skin tissue shown by tissue blot hybridization and immunohistochemistry. Expression of OATP-A and -C was not detected in any of the keratinocyte samples. In contrast, liver tissue showed a significant expression of OATP-A and -B as well as OATP-C, a weak expression of OATP-D and no expression of OATP-E. These data revealed that NHEK express a specific profile of transporters involved in drug influx. Using a newly developed uptake-transport assay, uptake of known and well characterized OATP substrates like estradiol-17β-glucuronide and estrone sulfate was inhibited in NHEKs by specific inhibitors such as taurocholate, verifying the functional capacity of the expressed OATPs. Human dermal fibroblasts seem to have a lower influx transport activity for estradiol-17β-glucuronide which correlates with the immunohistological data.
Even though the substrate specificity of the OATP isoforms is only partially known till now, our findings support the concept that uptake of large organic cations like drugs in keratinocytes is an active transport process mediated by members of the OATP family.

P186

Influence of different cyclodextrins on proliferation of HaCaT keratinocytes measured by means of bioluminescence and fluorometric assays

U. Hipler[1], P. Elsner[1], C. Hipler[1], U. Wollina[2]

[1]Klinik für Dermatologie und dermatologische Allergologie, D-07740 Jena
[2]Universiät Dresden, Klinik für Dermatologie, 01076 Dresden, Deutschland

Objectives: The influence of α,β-and γ-cyclodextrins on the proliferation of HaCaT keratinocytes has been studied by bioluminescence ATP measurements and fluorometric DNA quantitation. The aim of the study was the finding of non-cytotoxic cyclodextrin concentrations.
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).The HaCaT cells were incubated in a 96-well microplate with the different cyclodextrins in several concentrations (1 %,0.5 %,0.1 %) 24 h and 48 h, respectively. The determination of proliferation was carried out on the basis of the bioluminescence assay ATPLiteTM-M (Packard Bioscience B.V.,Niederlande) 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: Cyclodextrins in concentrations of 0.5 % and 0.1 % (incubation time 24 h) can generate a proliferation of HaCaT cells, while the higher concentrated (1%) cyclodextrins inhibit the proliferation of HaCaT cells after 48 h incubation.
Conclusions : The use of cyclodextrins in concentrations lower than 0.5 % seems to be harmless also for applications on the skin.

P187

Corticosteroids but not pimecrolimus induce apoptosis in murine langerhans cells in vivo

W. Hoetzenecker[1], R. Ecker[2], E. Kowalsky[3], J. Meingassner[3], G. Stingl[1], A. Stuetz[3], A. Elbe-Buerger[1]

[1]DIAID, Department of Dermatology, University of Vienna Medical School, VIRCC, 1230 Wien, Oesterreich
[2]Competence Center for Bio Molecular Therapeutics, 1230 Wien, Oesterreich
[3]Novartis Research Institute, 1230 Wien, Oesterreich

Pimecrolimus (Elidel®) is a novel anti-inflammatory drug that has recently been introduced into the market for the topical treatment of atopic dermatitis. It is a cell-selective inhibitor of inflammatory cytokine synthesis in T cells and mast cells. Given the importance of dendritic cells for immune functions, we investigated the effect of pimecrolimus on Langerhans cells (LC) in comparison with corticosteroids (CS). BALB/c mice were treated twice on one day on both sides of the ears with 20 µl ethanol (vehicle control) or ethanolic solutions of the test compounds at their clinically used concentrations: 1% hydrocortisone (HC), 0.05% clobetasol propionate (CL) and 1% pimecrolimus. 24 h after the last application, we observed by flow cytometry an upregulation of Fas (CD95), a TNF receptor family protein that transmits an apoptotic cell death signal, on LC treated with CS (HC and CL: 44 and 80% Fas^+ LC, respectively). Fas expression in LC of pimecrolimus-treated mice was comparable to controls (~ 30%). In accordance with these data, 48 h after the last application enhanced caspase-3 activity was noted in LC of CS-treated mice, whereas no activity was observed in LC of pimecrolimus- or vehicle-treated animals. To identify MHC class II^+ LC with fragmented DNA (cells in late apoptosis) we applied the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) technique to epidermal sheets. Using confocal laser scanning microscopy we detected 72 h after the last application 2% $TUNEL^+$ LC in control and pimecrolimus-treated skin but 12 and 23% $TUNEL^+$ LC in the HC and CL-treated skin, respectively. In summary our results show that CS but not pimecrolimus induce apoptosis of LC. This confirms that pimecrolimus has a more selective mode of action supporting a higher safety of topical pimecrolimus in the treatment of inflammatory skin diseases as compared to CS.

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FcγRIII receptor expression in cultured human keratinocytes with response to interferon γ treatment .

K. Cauza[1], A. Grassauer[1], G. Hinterhuber[1], R. Horvat[2], K. Rappersberger[3], K. Wolff[1], D. Foedinger[1]

[1]Department of Dermatology, Division of General Dermatology, University of Vienna Medical School, 1090 Wien, Oesterreich
[2]Dept. of Clinical Pathology, University of Vienna Medical School, A-1090 Wien
[3]Krankenanstalt Rudolfstiftung, Department of Dermatology, A-1030 Wien

Human keratinocytes actively participate in inflammatory and autoimmune reactions of the skin. They are able to produce a broad range of cytokines and interact with resident or infiltrating immunocompetent cells. Immunocompenent cells are to a great extent involved in immmunomodulatory functions via Fcγ receptors. We asked whether human keratinocytes might contribute to immunologic processes in the skin via Fcγ receptors. At the mRNA level, we show that human keratinocytes express specific mRNA for all three classes of Fcγ receptors, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16). Interferon γ is a known stimulator for FcγRI and FcγRIII in human leucocytes. We tested its stimulatory effects on expression of Fcγ receptors specific mRNA in cultured human keratinocytes by incubating for 10 hours with recombinant human interferon γ and detected significant upregulation of FcγRIII mRNA for both known subclasses FcγRIIIA and FcγRIIIB. At the protein level, we demonstrate expression of FcγRIII by performing immunofluorescence experiments showing a fine granular pattern at the cell surface of cultured keratinocytes whereas interferon γ treated keratinocytes show a more prominent granular signal on keratinocytes indicating upregulated protein expression. In immunoblotting experiments, anti-FcγRIII antibody shows a specific reactivity for a polypeptide of 50-65kDa in lysates of treated and untreated keratinocytes. Summarizing our data, we demonstrate mRNA expression of all three classes of Fcγ receptors in cultured human keratinocytes, interferon γ dependent upregulation of FcγRIII mRNA for both known subclasses A and B and FcγRIII protein expression by immunofluorescence and immmunoblotting experiments.

P189

The Thy1 / Thy-1-ligand-interaction is involved in binding of melanoma cells to activated Thy-1-positive microvascular endothelial cells.

A. Saalbach[1], A. Wetzel[2], U. Haustein[1], M. Sticherling[2], U. Anderegg[1]

[1]Sächsische Akademie der Wissenschaften zu Leipzig, 04103 Leipzig, Deutschland
[2]Universitätshautklinik Leipzig, Experimentelle Dermatologie, 04103 Leipzig, Deutschland

Cell adhesion plays a central role in metastasis of tumour cells as well as inflammation. Cell adhesion mediated by specific adhesion molecules direct and regulate the invasion of cells in certain tissues.
In recent studies, we characterized the human Thy-1 molecule as an inducible activation associated cell adhesion molecule on human dermal microvascular endothelial cells (HDMEC). Thy-1 expression has never been detected on resting endothelial cells in situ and in vitro. The Thy-1-ligand was detected on granulocytes and monocytes. Our experiments proved the involvement of the Thy-1 / Thy-1-ligand interaction in binding of PNC and monocytes to activated Thy-1-positive HDMEC. The strong connection of inflammatory events and Thy-1 expression suggests a specific role during binding of monocytes and PNC to activated endothelium establishing an event-specific interaction with PNC and monocytes from peripheral blood vessels during inflammatory responses or cell activation processes.
Furthermore, we could demonstrate a strong expression of Thy-1 on endothelial cells in tissue section of melanoma. In vitro, an induction of Thy-1 expression on HDMEC was seen after stimulation with melanoma cell derived soluble mediators. The Thy-1-ligand was found on different melanoma cell lines in vitro and on melanoma cells in situ. In cell adhesion assays the involvment of the Thy-1 / Thy-1-ligand interaction in binding of melanoma cells to activated Thy-1-positive HDMEC was shown.
Taken together, the investigation of Thy-1 and Thy-1 ligand expression might allow a more detailed insight in the direction and regulation of the invasion of cells in tissue with inflammation as well as in metastasis of certain tumor cells.

P190

Mechanism of PUVA induced growth arrest in human dermal fibroblasts: relevance of karyoplasts or cytoplast?

W. Ma[1], M. Wlaschek[1], L. A. Schneider[1], C. Hommel[1], K. Scharffetter-Kochanek[1]

[1]Dept. of Dermatology, Univ. of Ulm, 89081 Ulm, Deutschland

PUVA treatment as a combination of psoralen incorporation plus ultraviolet A irradiation is widely used in the treatment of a variety of hyperproliferative skin disorders. Recently, we have shown that following a single PUVA treatment human dermal fibroblasts underwent a long-term senescence-like growth arrest. However, the molecular mechanisms underlying this growth arrest are not known in detail. Nuclear effects as well as membrane damages have been attributed to be responsible.To address the question whether nuclear or membrane related damage is responsible for the long-term growth arrest, we performed cell fusion experiments using karyoplasts of PUVA-treated fibroblasts and enucleated cytoplasts of normal fibroblasts as well as vice versa. Fibroblasts were stably transfected with GFP and neomycin as marker genes, and treated with PUVA. At different time points post PUVA karyoplasts expressing GFP and neo^r were fused with cytoplasts of parental fibroblasts labeled with latex beads. Hybrids were selected in G418 containing medium and the successfully fused cells showed GFP expression and included latex beads. Similarly, cytoplasts of PUVA-treated cells were stained with Hoechst-33342 before fusion to label contaminating nuclei after fusion and fused with karyoplasts of normal fibroblasts. Results showed that hybrids with cytoplasts or karyoplasts from PUVA-treated fibroblasts taken up to one week post treatment died after 5 days. Hybrids with PUVA-treated cytoplasts and karyoplasts taken at two to four weeks post treatment showed remarkable differences. Hybrids with PUVA karyoplasts from cells at 4 weeks after treatment proliferated for up to 17 days after fusion. In contrast, hybrids with cytoplasts from PUVA treated cells did not die but were temporarily growth arrested for up to 12 days after fusion. From these experiments we conclude that PUVA treatment induces damages in both the nuclear (DNA) and the cytoplasmic compartment. We hypothesize that cytoplast effects are less damaging compared to nuclear effects by PUVA. In addition, factors in the cytoplast seem to have an ability to redirect cell to a proliferating or growth arrest state.

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The ectodomain of the transmembrane collagen XVII alters keratinocyte motility.

C. Franzke[1], K. Tasanen[2], B. Kerstin[3], F. Echtermeyer[3], L. Bruckner-Tuderman[1]

[1]Univ. of Münster, Dermatology, 48149 Münster, Deutschland
[2]Univ. of Oulu, Dermatology, Fin-90220 Oulu, Finland
[3]Univ. of Münster, Pathobiochemistry, 48129 Münster, Deutschland

Collagen XVII belongs to the novel family of transmembrane collagens and represents a structural component of the hemidesmosomes. It exists in two forms, as a 180 kDa type II transmembrane protein and as a soluble 120 kDa form, which corresponds to the extracellular collagenous domain of collagen XVII. We have previously shown that the release of the ectodomain from keratinocyte surface was catalyzed by at least three members of the ADAM family, whereas the cleavage of the collagen XVII molecule occurs within the NC16A domain. Time chase experiments with biotinylated keratinocytes revealed that the ectodomain was detectable within minutes and showed high stability in the medium for more than 72 hours. The use of domain specific antibodies demonstrates that the authentic shedding product contains at least a part of the NC16A domain and the full C-terminus of the collagen XVII molecule. Since only little was known about the physiological function of the shed ectodomain, the influence on cell motility was investigated. Haptotactic migration assays with collagen I coated transwell chambers revealed that collagen XVII transfected COS cells showed a 2.5-fold increased motility compared to empty vector transfected cells, whereas transfection with a not shed NC16A deletion construct of collagen XVII induces a significant increased transmigration rate. Transfection of HaCaT cells with cDNA encoding for TACE, ADAM-10, and ADAM-9 leads to an overproduction of collagen XVII-ectodomain into the culture medium. In vitro wound closure assays revealed that all three ADAM-transfected cells showed a markedly reduced cell motility after 22 hours. In addition, in vitro scratch assays with normal human keratinocytes were performed. The addition of 1 nM purified authentic collagen XVII ectodomain caused decreased keratinocyte migration after 16 hours. Therefore, it can concluded that the shed collagen XVII ectodomain is involved in the regulation of keratinocyte motility in physiological and pathological processes.

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Epidermal Ceramide Metabolism in Aged Skin

J. M. Jensen[1], S. Winoto-Morbach[2], M. Schunck[1], C. Neumann[1], M. Foerl[1], S. Schuetze[2], E. Proksch[1]

[1]Christian-Albrechts-Universitaet, Dermatologie, 24105 Kiel, Deutschland
[2]Christian-Albrechts-Universitaet, Immunologie, 24105 Kiel, Deutschland

Epidermal ceramides are the major component of stratum corneum lipids, which are responsible for skin barrier function. In aged humans, as well as in senescent mice, barrier repair capacity is significantly reduced. However, basal skin barrier function is normal in aged skin. In the present study, we examined the activity of the ceramide providing enzymes, ceramide synthase and acid sphingomyelinase in the epidermis of aged (>18 months) compared to young hairless mice (<3 months). Furthermore, we determined the catabolic acid ceramidase. The ceramide synthase activity assay was performed using ^{14}C-labeled palmitoyl-CoA as described in J Biol Chem 275: 30344-54, 2000. Acid sphingomyelinase activity, using ^{14}C-sphingomyelin, was determined as shown in J Clin Invest 104: 1761-70, 1999. For assaying katabolic acid ceramidase, ^{14}C-palmitoyl-sphingosine was used as substrate according Adv Exp Med Biol 477: 305-15, 2000. Ceramide synthase activity was 40% reduced in the epidermis of senescent in comparison to young mice. Acid sphingomyelinase showed unchanged activity in the uppermost epidermal layers, but reduced activity of 39% was found in the inner epidermis in aged compared to young controls. Ceramide catabolizing acid ceramidase remained unchanged, too. In summary, the reduced ceramide synthase activity and the lower activity of sphingomyelinase in the lower layers of the epidermis correlate with the reduced capacity in permeability barrier repair in senescent skin. Acid sphingomyelinase in the outer layers of the epidermis and acid ceramidase are most important for skin barrier homeostasis under basal conditions and are unchanged even in age.

P193

Inhibition of p53-activity in the skin downregulates Caspase-14 expression and is associated with an impaired terminal differentiation of keratinocytes

M. Mildner[1], C. Ballaun[1], P. Mrass[1], S. Karner[1], E. Tschachler[1,2]

[1]Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, 1160 Wien, Oesterreich
[2]Centre de Recherches et d'Investigations Epidermiques et Sensorielles (C.E.R.I.E.S.), 29521 Neuilly, France

The tumor suppressor gene p53 plays a key role in the regulation of cellular responses to DNA damage that lead to apoptosis or cell cycle arrest and DNA-repair. However, the involvement of p53 in the differentiation of the skin is still poorly understood.
In this study we have investigated the effect of a chemical inhibitor of p53, α-Pifithrin, on keratinocyte differentiation and Caspase-14 expression in skin-equivalents.
After incubation of skin-equivalents with α-Pifithrin for 7 days we found a strong downregulation of p53 mRNA and protein, as well as the p53 downstream element, $p21^{WAF1/Cip1}$. H&E staining of these skin-equivalents revealed a parakeratotic phenotype. When analyzing expression of Caspase-14, which is upregulated and activated during regular terminal keratinocyte differentiation, we found a strong reduction of Caspase-14 protein and m-RNA by α-Pifithrin. To study the effect of p53 inactivation on caspase 14 expression *in vivo*, we studied skin biopsies of Bowen´s disease, actinic keratosis and condyloma accuminata, in which p53 is inactivated by mutations and HPV respectively, for Caspase-14 expression. In lesional skin of all three diseases Caspase-14 expression was strongly reduced whereas it was not affected in adjacent skin. Our additional finding that the Caspase-14 gene contains a functional p53 binding site in the intron-1, strongly suggests that the effect of p53 inactivation on Caspase-14 expression is a direct one. In conclusion, our results suggest that p53 plays an important role in the terminal differentiation of epidermal keratinocytes, and that it is directly involved in the regulation of Caspase-14 during this process.

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Human Leukocyte Elastase induces Calcium-Influx in Keratinocytes independent from Protease-activated-Receptor Signaling

J. Wingertszahn[1], U. Meyer-Hoffert[1], O. Wiedow[1]

[1]Department of Dermatology UK Kiel, 24105 Kiel, Deutschland

Psoriasis is characterized by a cellular infiltrate of neutrophils into the epidermis. Neutrophils release serine proteases like human leukocyte elastase (HLE) at the site of inflammation. We were able to show in previous studies that HLE induces keratinocyte hyperproliferation in vivo and in vitro. The molecular mechanism remained speculative. As it is known that some proteases like thrombin, trypsin or mast cell tryptase are capable of inducing cell proliferation via the family of protease-activated receptors (PAR), a group of G-protein linked seven transmembrane receptors, we were interested whether HLE is able to induce cell signaling in keratinocytes by activation of PARs.
In this study we were able to observe a transient Ca-influx in FURA-loaded HaCaT keratinocytes by single-cell video-imaging microscopy after stimulation with 100 nM HLE. An increase in intracellular calcium was observed approximately 300 sec after stimulation, which reached normal low calcium levels after approximately 1000 sec. The Ca-influx was dependent on proteolytic activity as demonstrated by inhibition with the HLE inhibitor elafin (1 µM). Preincubation with the selective SERCA-inhibitor thapsigargin abolished the HLE-mediated Ca-influx demonstrating that the signal was dependent from internal Ca-stores. It was possible to induce Ca-influx after activation with PAR1 and PAR2 agonists. After pre-activation with trypsin (100nM) cells exhibited a Ca-influx after HLE and vice versa. Preincubation with pertussis toxin to deactivate G-protein signaling, inhibited Ca-influx by PAR1, PAR2 agonists and trypsin, however, the HLE induced Ca-influx was not affected by pertussis toxin preincubation. We conclude from these findings that HLE induces Ca-influx in keratinocytes not by PAR-mediated activation. Further investigations have to elucidate the molecular mechanism and whether this signaling pathway leads to HLE driven hyperproliferation in keratinocytes.

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Expression of Aryl hydrocarbon Receptor (AhR) and Aryl hydrocarbon Receptor Repressor (AhRR) in human Keratinocytes and dermal Fibroblasts

E. Fritsche[1], J. Abel[1]

[1]IUF, Exp. Tox., 40225 Düsseldorf, Deutschland

The Aryl hydrocarbon Receptor (AhR), Aryl hydrocarbon Receptor Repressor (AhRR) and their common cofactor ARNT (Aryl hydrocarbon Receptor Nuclear Translocator) are members of the superfamily of bHLH/PAS transcription factors which are important in development, growth, and differentiation. The AhR is an intracellular, ligand activated mediator of xenobiotic signaling. Ligands such as TCDD bind to the receptor with high affinity, and after binding the receptor-ligand complex translocates into the nucleus. There, the partner ARNT dimerizes to the complex and initiates gene activation via interactions with XRE-elements that are located within the promoter of drug metabolizing enzymes. The AhRR has been found to repress AhR function by competition over ARNT and subsequent downregulation of transcription.
Recently it was reported that dermal fibroblasts fail transcriptional activation of drug metabolizing enzymes by TCDD (JBC (1999) 274:13522-13518). In order to evaluate possible mechanisms involved we started to analyze the expression of the AhR and AhRR in human keratinocytes, HaCat cells and dermal fibroblasts.
RNA was isolated from primary human keratinocytes, HaCat cells and primary human dermal fibroblasts and real time RT-PCR was performed using a LightCycler Instrument (Roche). Within each run, a PCR product specific copy number standard was amplified to be able to compare absolute amounts of transcripts inbetween different runs.
Our results show that keratinocytes, HaCat cells and fibroblasts exhibit similar levels of AhR mRNA. In contrast, the expression of AhRR mRNA significantly differed between keratinocytes and HaCat cells on one side and fibroblasts on the other side. Accordingly, the expression of AhRR mRNA is approx. 20 times higher in fibroblasts than in HaCat cells. AhRR mRNA expression varies up to 15 fold in keratinocytes from different individuals. These levels were 20 to 300 times lower than in fibroblasts.
In summary, our findings suggest that the high expression of AhRR in fibroblast may be responsible for the reported impaired xenobiotic metabolism as a result of enhanced recruitment of ARNT. Studies to test this hypothesis are ongoing.

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Uptake of silica DQ12 by fibroblasts and endothelial cells in vitro

U. Anderegg[1], G. Fitzl[2], J. Grosche[3], J. Kacza[4], U. Haustein[1,5]

[1]Sächsische Akademie der Wissenschaften zu Leipzig, 04103 Leipzig, Deutschland
[2]Institut f. Anatomie der Universität Leipzig, 04103 Leipzig, Deutschland
[3]Paul-Flechsig-Institut f. Hirnforschung der Universität Leipzig, D-04109 Leipzig
[4]Institut f. Veterinäranatomie der Universität Leipzig, 04103 Leipzig, Deutschland
[5]Universitätshautklinik Leipzig, Experimentelle Dermatologie, D-04103 Leipzig

Background: Silica (<5 µm) is able to induce systemic sclerosis (SSc) that is indistinguishable from idiopathic disease. Therefore, silica can serve as a model to investigate initial processes during the pathogenesis of SSc - especially skin fibrosis. In cell culture models we demonstrated that relevant cell types are activated by silica in a manner that reflects known phenomena from SSc.
Objective: However, the mechanism of cell-crystal-interaction remained unclear for fibroblasts and endothelial cells while the uptake of silica crystals by monocytes had been shown before.
Methods: Using laser scanning microscopy (LSM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) we tried to identify silica crystals (DQ12) within or attached to fibroblast or endothelial cells cultured on semi-permeable membranes.
Results: Silica crystals scatter the laser beam used in laser scanning microscopy very much, so we could not clearly show the internalization of silica using this method. In electron microscopy the cutting procedure is limiting because the crystals are not cut and often destroy the probe. However, the photos presented here, show that silica-treated fibroblasts as well as endothelial cells had included structures remembering the crystals. These typical structures were absent in control cells cultured without silica. Scanning Electron Microscopy (SEM) was able to prove the hypothesis of silica uptake by fibroblasts and HDMEC.Using SEM we could demonstrate that crystals are within the cultured cells under the cell membrane. The cells are alive during culture demonstrated earlier. The photos demonstrate that fibroblasts and HDMEC can uptake large crystals and more than one crystal per cell.
Conclusion: From these data we conclude that fibroblast and endothelial cells might be able to internalize the silica crystals. Therefore the silica-mediated gene expression of these cells might be due to outsight-in as well as insight-in mediated mechanisms.

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HAX-1 identified by differential display RT-PCR is overexpressed in lesional psoriasis

A. Mirmohammadsadegh[1], U. Tartler[1], A. Bär[1], G. Michel[1], T. Ruzicka[1], U. R. Hengge[1]

[1]Heinrich Heine-Universität Düsseldorf, Hautklinik, 40225 Düsseldorf, Deutschland

Besides an inflammatory infiltrate, the main characteristic of psoriasis is a massive disturbance of keratinocyte differentiation leading to epidermal hyperproliferation. This could be due to both excess mitogenic and resistance to apoptotic signals. In an attempt to depict a maximum number of pathologically dysregulated genes we performed mRNA differential display analysis (DDRT-PCR) comparing mRNA fingerprints from lesional psoriatic skin with non-lesional and healthy skin. Differentially expressed products were isolated, cloned and sequenced. One of these so far unidentified cDNAs was identified as HAX-1 by searching the EMBL sequence database using FASTA. In our studies overexpression of the gene in lesional vs. nonlesional psoriatic skin was verified at the mRNA level by Northern blot and in situ hybridization as well as at the protein level by immunohistochemistry. HAX-1 was initially described in the context of downstream signaling of antigen receptor-coupled tyrosine kinases in lymphocytes exhibiting properties as an inhibitor of apoptosis. In psoriasis, disturbed epidermal differentiation could be due to prolonged expression of HAX-1 and hence prolonged resistance to terminal differentiation and apoptosis. Our data provide evidence that this protein might have a role in the control of the epidermal differentiation pathway by delaying programmed cell death.

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Peroxisome proliferator-activated receptor-α agonists effectively inhibit vascular endothelial growth factor receptor-2 gene expression by endothelial cells

M. Meissner[1], M. Stein[1], K. Reisinger[1], R. Kaufmann[1], J. Gille[2]

[1]Klinikum der J.W. Goethe-Universität, Department of Dermatology, 60590 Frankfurt am Main, Deutschland
[2]Max-Planck-Institut für Physiologische und Klinische Forschung, Department of Molecular Biology, 61231 Bad Nauheim, Deutschland

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, mainly implicated in the regulation of lipid and glucose homeostasis. In addition, natural and synthetic PPAR agonists may control inflammatory processes by inhibition of distinct proinflammatory genes. As signaling via the vascular endothelial growth factor receptor-2 (VEGFR2) pathway is critical for angiogenic responses during chronic inflammation, we explored whether anti-inflammatory effects of PPAR ligands could be mediated in part through diminished VEGFR2 expression. In this study, PPAR-α agonists are found (WY-14643, fenofibrate) to potently inhibit endothelial VEGFR2 expression, whereas predominant PPAR-γ ligands (ciglitazone, 15d-PGJ2) remained without discernable effects. Time- and concentration-dependent inhibition is demonstrated both at the level of protein (FACS, Western blot analysis) and mRNA VEGFR2 expression (Northern blot analysis). Inhibitory effects of PPAR-α agonists on transcriptional activity of the VEGFR2 promoter is conveyed by an element located between bp -60 and -37 that contains two adjacent consensus Sp1 transcription factor binding sites. Constitutive Sp1-dependent DNA binding to this sequence is decreased by PPAR-α treatment, indicating that PPAR-α activation may interfere with Sp1-dependent VEGFR2 transcription. Since PPAR-α agonists greatly attenuate VEGF-driven capillary-like network formation as VEGFR2-dependent endothelial cell function, anti-inflammatory effects of PPAR-α ligands may be likely mediated in part via reduced VEGFR2 expression.

P199

Fluid shear stress-induced transcriptional activation of the vascular endothelial growth factor receptor-2 gene requires Sp1-dependent DNA binding

C. Urbich[1], M. Stein[2], K. Reisinger[2], R. Kaufmann[2], S. Dimmeler[1], J. Gille[3]

[1]Klinikum der J.W. Goethe-Universität, Molecular Cardiology, Department of Internal Medicine IV, 60590 Frankfurt am Main, Deutschland
[2]Klinikum der J.W. Goethe-Universität, Department of Dermatology, 60590 Frankfurt am Main, Deutschland
[3]Max-Planck-Institut für Physiologische und Klinische Forschung, Department of Molecular Biology, 61231 Bad Nauheim, Deutschland

Hemodynamic forces play a fundamental role in the regulation of endothelial cell survival. As signaling via the vascular endothelial growth factor (VEGF) receptor-2 pathway has been previously demonstrated to impact endothelial cell survival, we hypothesized that laminar shear stress may facilitate survival in part by inducing VEGF receptor-2 expression. This study shows a time- and dose-dependent upregulation of endothelial VEGF receptor-2 expression by fluid shear stress in microvascular and large-vessel derived endothelial cells. A functional analysis of the 5'-regulatory region of the VEGF receptor-2 promoter localized the shear stress-response element to a sequence between bp -60 and -37 that encompasses two adjacent consensus Sp1 transcription factor binding sites. Constitutive and shear stress-inducible Sp1-dependent complexes are bound to this element, indicating that fluid shear stress-induced transcriptional activation of the VEGF receptor-2 gene requires Sp1-dependent DNA binding. Together, these results suggest that biomechanical stimulation may lead to endothelial cell survival by upregulating VEGF receptor-2 expression.

P200

Interleukin-1 enhances UVB-induced apoptosis by different mechanisms

D. Kulms[1], B. Poeppelmann[1], T. Schwarz[1]

[1]University Muenster, Department of Dermatology, D-48149 Muenster

Treatment of KB cells (epidermoid carcinoma cell line) with the cell death ligands FasL or TRAIL and irradiation with UVB, respectively, induces apoptotic cell death. Prestimulation of KB cells with interleukin 1 (IL-1) which activates the transcription factor nuclear factor κB (NFκB) was shown to protect cells from FasL- and TRAIL-induced apoptosis via upregulation of the inhibitor of apoptosis proteins (IAP) c-IAP and x-IAP. In contrast, UVB-induced apoptosis was significantly enhanced upon prestimulation with IL-1 and coincided with a strong NFκB-mediated release of the pro-apoptotic cytokine tumor necrosis factor-α (TNFα) in the range of 100 pg/ml. No TNFα release occurred upon treatment of KB cells with IL-1 plus either one of the death ligands. Surprisingly, costimulation of cells with 100 pg/ml TNFα and UVB resulted only in a rather weak enhancement of UVB-induced apoptosis, indicating that other mechanisms besides TNFα release have to be involved in the apoptosis enhancing effect of IL-1. Semiquantitative PCR analysis revealed that NFκB-dependent transcription of c-IAP and x-IAP as well as of FLIP (FLICE inhibitory protein), another anti-apoptotic protein, was significantly downregulated by UVB radiation. This downregulation seemed to be critically dependent on UVB-induced DNA-damage, since it could be reversed by addition of liposomes containing the DNA repair enzyme photolyase. Addition of IL-1 to UVB-exposed cells caused a further downregulation of the anti-apoptotic proteins, thus explaining the enhanced death rate of UV-exposed cells in the presence of IL-1. Together, these data demonstrate that IL-1 enhances UVB-induced apoptosis in several ways, first by inducing the secretion of TNFα and second by supporting the downregulation of anti-apoptotic proteins. This further indicates that the combination of IL-1 and UVB can cause transcriptional up- (TNFα) and downregulation (anti-apoptotic proteins) of different genes in parallel and thereby contribute to the same biological effect, i.e. enhancement of apoptosis.

P201

The 97 kDa (LABD97) and 120 kDa (LAD-1) Linear IgA Disease Antigens Have Different N-Termini

Y. Hirako[1], Y. Nishizawa[2], C. Sitaru[1], E. Butt-Dörje[3], K. Owaribe[2], D. Zillikens[1]

[1]University of Würzburg, Department of Dermatology, 97080 Würzburg, Deutschland
[2]Nagoya University, School of Human Informatics, 464-8601 Nagoya, Japan
[3]University of Würzburg, Institute of Clinical Chemistry and Pathobiochemistry, 97080 Würzburg, Deutschland

Type XVII collagen is a hemidesmosomal transmembrane glycoprotein. Several subepidermal blistering diseases are associated with an autoimmune response to this molecule, including linear IgA disease (LAD). Two proteolytic fragments of type XVII collagen are the major antigenic targets in LAD, including epidermis-derived 97 kDa (LABD97) and keratinocyte-derived 120 kDa (LAD-1) proteins. Interestingly, while LAD sera react with both of these fragments, they show poor binding to full-length type XVII collagen suggesting the generation of novel autoantigenic epitopes on LABD97 and LAD-1 induced by the cleavage. Both fragments were assumed to have the same N-terminus within the membrane-proximal NC16A domain of type XVII collagen. However, in contrast to LABD97, the N-terminus of LAD-1 has not yet been identified. To address this issue, in a first set of experiments, we immunoadsorbed serum from a rabbit, immunized against the recombinant NC16A domain, using 10 overlapping fragments covering the entire domain [amino acids (aa) 490 to 565]. Rabbit IgG purified against a peptide covering aa 514-532 retained reactivity with LAD-1, while antibodies specific to a fragment covering aa 508-521 lost this reactivity, indicating that the N-terminus of LAD-1 localizes within a stretch corresponding to aa 521-532. To determine the exact cleavage site, we performed N-terminal sequencing of LAD-1 affinity-purified from conditioned medium of cultured keratinocytes. This revealed leucine at aa position 524 as the N-terminus of the polypeptide. Interestingly, this position was 7 aa upstream of aa position 531 that had been reported as the N-terminus of LABD97. Finally, mass spectrometry of trypsin-digested fragments of LAD-1 demonstrated the presence of fragments containing aa 528 (alanine), confirming that the N-terminus of LAD-1 is different from that of LABD97. These data suggest that LAD-1 and LABD97 are generated through different proteolytic processes from type XVII collagen. Characterization of the N-terminus of LAD-1 should facilitate future studies on the shedding of the type XVII ectodomain and the analysis of epitopes on LAD-1 induced by the cleavage.

P202

A cytotoxic metabolite from *Malassezia furfur*: isolation and chemical identification as malassezin

M. Podobinska[1], H. J. Kraemer[2], A. Bartsch[3], W. Steglich[3], P. Mayser[1]

[1]Justus-Liebig-Universität Giessen, Zentrum für Dermatologie und Andrologie, 35385 Giessen, Deutschland
[2]Justus-Liebig-Universität Giessen, Klinische Pharmakologie, D-35385 Giessen
[3]Ludwig-Maximilians-Universitaet München, Department Chemie, D-81377 München

Tryptophan as sole nitrogen source induces the synthesis of fluorochromes and pigments in cultures of *Malassezia (M.) furfur*. In lesions of pityriasis versicolor depigmented areas are common. Whether this depigmentation is due to inhibition of melanogenesis or to toxic influences on melanocytes is under discussion. We isolated a metabolite of *M. furfur*, which was chemically identified as malassezin, a potent inductor of the arylhydrocarbon receptor (G. Wille et al, Bioorg. Med. Chem. 2001, 9, 955-960). *M. furfur* (CBS1878) was grown for 14 days on a special medium (30ml Tween 80, 3g Trp, 20g Agar dissolved in 1L of water) at 30ºC. The agar was extracted with ethyl acetate. The resulting strongly coloured extract was exposed to cultures of primary human melanocytes. A dose-dependent toxic influence on the cells could be detected, observable by retraction of pseudopodia and formation of nuclear bodies. Further separation of the extract by Sephadex column chromatography (Sephadex LH20, elution with methanol) yielded 8 fractions, which showed specifically the observed toxicity in fraction 2. It was submitted to preparative TLC (eluent: toluene:ethyl formate:formic acid;10:5:3), resulting in 16 subfractions with the toxic effect in one fraction with Rf = 0.38. Following preparative RP-HPLC (linear gradient elution with acetonitrile/water 0-100%; RP8) revealed the toxic influence corresponding with a single substance.The substance was identified by high-resolution MS and NMR spectroscopy as malassezin [2-(1H-indol-3-ylmethyl)-1H-indole-3-carbaldehyde], a known indole metabolite from M. furfur. Malassezin is a potent inductor of the Ah-receptor (EC_{50}=1.57 µM). In cultured melanocytes, malassezin exhibited dose dependent the cellular changes described above, with an approx. IC_{50} of 5 µM. Vitality (trypan blue exclusion) as well as proliferation rate (MTT-test) were only slightly influenced. We postulate a major influence of malassezin on cytoskeletal rearrangement in melanocytes and thus an inhibitory influence on melanin transport. Malassezin may therefore contribute to the depigmentation in areas of pityriasis versicolor.

P203

Degradation of Bullous Pemphigoid Antigen 180 is Inhibited by Trehalose During Desiccation and Freezing

E. Schmidt[1,2], A. Kromminga[3], M. Kürschner[1], V. Sukhorukov[1], H. Zimmermann[4], A. D. Katsen[4], D. Zillikens[2], E. Bröcker[2], U. Zimmermann[1]

[1]University of Würzburg, Dept. of Biotechnology, 97080 Würzburg, Deutschland
[2]University of Würzburg, Dept. of Dermatology, 97080 Würzburg, Deutschland
[3]Institute of Immunology, Clinical Pathology, and Molecular Medicine, 22339 Hamburg, Deutschland
[4]Fraunhofer Institute for Biomedical Engineering, Dept. of Low Temperature Biophysics, 66386 St. Ingbert, Deutschland

Bullous pemphigoid antigen 180 (BP180) is targeted by autoantibodies in a variety of subepidermal blistering skin diseases. We have recently developed a simple, highly specific and sensitive immunofluorescence (IF) assay for the detection of circulating antibodies against BP180. This novel assay involves the expression of full-length (FL) BP180 in Sf21 insect cells that are then subjected to IF microscopy after staining with anti-BP180 antibodies. Application of this assay as a routine diagnostic tool requires long-term storage of FL-BP180 which can result in substantial degradation of the antigen. Here, we show that the disaccharide trehalose, a natural cryo- and lyoprotectant, is capable of preserving the FL-BP180 antigen expressed in Sf21 insect cells under various storage conditions, including 40°C, room temperature, 4-8°C, -20°C, and -80°C. The protective effect was dose-dependent reaching maximum at about 200 mM trehalose. Trehalose was superior to other sugars or conventional cryoprotective agents (e.g., sucrose, myo-inositol, DMSO) in preventing degradation of the antigen. Trehalose inhibited degradation of both extra- and intracellular epitopes of FL-BP180. Interestingly, protection of the intracellular domain was only observed when trehalose was introduced into the cytosol. Trehalose significantly prolonged the storage time of FL-BP180 expressed in Sf21 insect cells, thus, enabling the routine use of the IF assay for the detection of serum antibodies.

P204

Localization of Bullous Pemphigoid Antigen 180 in Cultured Human Keratinocytes: Functionally Relevant Modification by the Binding of BP180-Specific Antibodies.

E. Schmidt[1], B. Wehr[1], C. Sitaru[1], Y. Hirako[1], E. Bröcker[1], D. Zillikens[1]

[1]Department of Dermatology, University of Würzburg, 97080 Würzburg, Deutschland

Bullous pemphigoid antigen 180 (BP180) is a hemidesmosomal transmembrane protein essential for the integrity of the dermal-epidermal junction. Its distribution in cultured keratinocytes depends on extracellular calcium (Ca^{2+}). Furthermore, BP180 has previously been shown to mediate, upon incubation with anti-BP180 antibodies, a specific signal transducing event that leads to the release of inflammatory mediators, such as IL-8 and tPA, from cultured normal human epidermal keratinocytes (NHEK). Here, the effect of antibodies to human BP180 on the distribution of this autoantigen in NHEK was investigated by confocal laser scanning microscopy. Antibodies to BP180 were obtained from BP patients and from a rabbit immunized against recombinant BP180; NHEH were grown in low (0.15 mM) or high (1.65 mM) Ca^{2+} medium. When NHEK, grown under low Ca^{2+} conditions, were treated with BP180-specific antibodies for 0.5 or 12 h, respectively, BP180 remained localized at the cell surface. NHEK grown in high Ca^{2+} medium and treated with antibodies to BP180 surface for 0.5 h showed the same cell surface staining, whereas this staining was greatly reduced in cells incubated for 12 h. Low and high Ca^{2+} conditions did not change expression of BP180 in NHEK as detected by immunoblotting. Interestingly, supernatant of NHEH, grown under high Ca^{2+} conditions, in contrast to medium from cells in low Ca^{2+}, showed no elevated levels of IL-8 and tPA after a 12 h treatment of cells with anti-BP180 IgG compared to medium from cells incubated with normal IgG. This study demonstrates that the Ca^{2+}-dependent distribution of BP180 is modified by the binding of antibodies to BP180. In addition, this effect was shown to be functionally relevant. Our data may help to further understand the complex pathomechanisms in blister formation of BP180-related autoimmune blistering skin diseases.

P205

Cutaneous inflammation induced by streptolysin O is mast cell-dependent

M. Magerl[1], M. Metz[1], I. Walew[2], J. Knop[1], S. Bhakdi[2], M. Maurer[1]

[1]University Hospital Mainz, Department. of Dermatology, D-55101 Mainz
[2]University Hospital Mainz, Institute of Medical Microbiology and Hygiene, 55101 Mainz, Deutschland

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 detect bacteria and how bacteria activate MC. Here, we asked whether bacterial toxins, i.e. streptolysin O (SLO) released by Streptococci contribute to MC activation after infection. To test whether SLO can induce MC degranulation ex vivo, murine peritoneal MC (pMC) were incubated with radiolabeled serotonin ([3H]5-HT, 2µCi/ml, 2h, 37ºC). Release was calculated as the percentage of total incorporated [3H]5-HT present in the cell supernatant 10min after stimulation with SLO (0.05-50µg/ml), Ca^{++} ionophore, or vehicle. SLO induced pronounced [3H]5-HT release from pMC (>40%) in concentrations as low as 5µg/ml, indicating that SLO tissue levels in Streptococci< infections can suffice to degranulate MC. Thus, we injected SLO (0.01-50µg/ml) or vehicle into ears of C57BL/6 mice and measured increases in skin thickness, a very sensitive parameter for extravasation induced by local MC degranulation. We found that SLO injections resulted in robust and dose dependent ear swelling responses (SLO 10µg/ml: 152±4µm vs. 63±11µm, $p<0.001$). Maximum increases in ear thickness were seen 1h after injection of SLO suggesting MC involvement. To test whether SLO induced inflammatory reactions are impaired in the absence of MC, we injected 20µl SLO (10µg/ml) or vehicle into the skin of genetically MC-deficient Kit^{W}/Kit^{W-v}-mice and normal Kit+/+ mice. Ear swelling in SLO-injected MC-deficient ears was markedly reduced as compared to normal ears (86±7µm vs. 136±9µm at 1h, $p<0.005$) and similar to those seen in vehicle treated ears (65±5µm at 1h). To prove that MC are required for SLO to elicit skin inflammation, we assessed SLO-induced ear swelling in Kit^{W}/Kit^{W-v}-mice that had, or had not, been reconstituted with MC. Notably, adoptive transfer of MCs restored significant swelling responses after injection of SLO in Kit^{W}/Kit^{W-v} ears, proving that SLO-induced inflammation is MC-dependent. Our findings suggest that bacterial toxins must not merely be viewed as virulence factors that harm the host, but may also function as crucial signals for the induction of MC-dependent innate immune responses to bact. inf.

P206

Automated Tracking of Cell Movements and Resolution of Cell-Cell Collisions in Three-dimensional Collagen Matrices

J. Moeller[1], B. Gottfried[2], C. Schlieder[2], O. Herzog[2], P. Friedl[1]

[1]Univ. of Würzburg, Dept. of Dermatology, 97080 Würzburg, Deutschland
[2]Univ. of Bremen, Technologiezentrum Informatik, 28334 Bremen, Deutschland

The migration of different cell types such as leukocytes and tumor cells within tissues is a fundamental process in physiologic and pathologic tissue reactions. However, the investigation and reconstruction of cell migration within 3D extracellular matrices in vitro and in vivo is limited by the lack of automated quantification methods that allow the reconstruction of cell paths from cell populations. We have developed a fully automated cell tracking device for the reconstruction of migration paths from 3D collagen matrices in front or inhomogeneous backgrounds independent of cell size and shape, sharpness of the boundary, shape change, and their migration velocity as well as cell-cell contacts. Best results for separation of cells from background (segmentation) were obtained by region growing from the center of preselected cells until a color gradient was reached. Noise reduction filtering and an object matching routine comparing the pixel mass from frame to frame was used to optimize segmentation stability upon object shape change and background variation, thereby increasing path precision. Overlapping paths and cell-cell contacts were resolved by a subtraction algorithm using mass changes for path separation. Compared to manual tracking as a "gold" standard automatic tracking resulted in high precision and reproducibility of path position, step lengths and frequencies (p=0.78 to 0.96; Mann-Whitney U-Test) for migrating ameoboid T cells as well as tumor cells. This automated device may be of value for quantitative detection of migrating cells in 3D extracellular matrix environments in basic research and drug development.

P207

p38 MAP Kinase and HSP27 Are Induced by Mechanical Pressure in Human Keratinocytes

M. Hofmann[1,2], A. Bernd[1], J. Bereiter-Hahn[2], R. Kaufmann[1], S. Kippenberger[1]

[1]JW Goethe Universitaet, Dermatologie, 60590 Frankfurt/M., Deutschland
[2]JW Goethe Universitaet, Arbeitskreis Kinematische Zellforschung, 60439 Frankfurt/M., Deutschland

Cells within human skin are permanently targeted by mechanical forces of different qualities. Mechanical stretching which is considered as a proliferation stimulus is presented e.g. in abdominal skin enlargement during pregnancy. Vice versa other locations like the palms of the hand or the foot soles are more prone to mechanical pressure. In this context mechanical pressure is thought to trigger cellular differentiation processes. Additionally, mechanical pressure is also discussed as a trigger factor of some skin diseases (Koebner phenomena). The underlying molecular signalling pathways which are involved in transduction of mechanical pressure are still enigmatic yet.
In the present in vitro attempt HaCaT cells were mechanically stimulated by insertion of a teflon weight into the culture dish (0.015N/cm2). Keratinocytes were analysed after different time intervals using SDS polyacrylamide gelelectrophoresis and western blotting for activation of members of the mitogen-activated protein kinases (MAPK). Mechanical pressure applied for a maximum time of 30 min showed a peak phosphorylation for p38 at 10 min. p44/42 MAPK was not phosphorylated by mechanical pressure stimulation. In order to further dissect the signalling cascade upstream and downstream regulators of p38 were examined. We demonstrate a transient activation of MEK3/6 upstream of p38. Downstream of p38 the phosphorylation of the small heat shock protein HSP27 could be shown. Phosphorylation of HSP27 is blocked by preincubation with p38 inhibitors (SB 209021 / SB 203580).
p38 MAPK is suggested to be involved in the cellular stress response because other established stimulators of p38 are osmotic stress, heat shock, and inflammatory cytokines. Our experimental results feature mechanical pressure as a new type of cellular stress which yields in p38 activation. As p38 plays an important role in the IL-8 induction we suggest mechanical pressure as a pro-inflammatory stimulus. These findings may contribute to the molecular understanding of mechanically triggered skin diseases like psoriasis and vitiligo.

P208

High-density oligonucleotide array analysis of interleukin-6 sensitive and resistant human melanoma cells

Z. Li[1], T. A. Luger[1], M. Böhm[1]

[1]University of Münster, Department of Dermatology and Ludwig Boltzmann Institute for Cell Biology and Immunobiology of the Skin, D-48149 Münster

Cytokine resistance is a well described phenomenon of advanced melanoma and has been established for interferons and interleukin-6 (IL-6). The molecular base for IL-6 resistance in melanoma still remains elusive. We performed high-density oligonucleotide array analysis of two representative human melanoma cell lines, WM35 (derived from an early primary tumor; IL-6 sensitive) and WM9 (derived from a metastasis; IL-6 resistant) stimulated with IL-6 for various time points (1, 3, 6 and 12 h). Hybridization of biotinylated cRNA samples was performed on HuGeneFL Affymetrix gene chips. Data evaluation, cleansing, extraction and analysis of the approximately 5600 genes was performed by the Gene Data Analyst vs.1 Expressionist software. The filter criteria included a minimum threshold of 100, normalization by the logarithmic mean and a quality setting of p<0,04. Only robust changes with a change factor of >3 were regarded as significant. IL-6 treatment of WM35 cells resulted in a time-dependent induction of several known IL-6 responsive genes such as the transcription factors NF-IL6beta, junB and c-fos, none of which being regulated in WM9 cells. In contrast, IL-6 treatment of WM9 cells led to an upregulation of proleukin-1beta and the chemokine IL-8, the latter a known growth factor for melanoma. By applying the above criteria, several novel IL-6-regulated functional gene clusters were identified in WM35 cells which included transcription factors (e. g. transducin-like enhancer protein, hypoxia-inducible factor 1alpha), cell cycle players (e. g. cdc2-related kinase), signal transduction components (e. g. dual-specificity phosphatase-5/hVH3/B23) and enzymes crucially involved in DNA synthesis (e. g. deoxyguanosine kinase). None of these genes was significantly altered by IL-6 in WM9 cells. Further studies are required to confirm these data at the protein level and to assess the relevance of the identified gene products with regard to IL-6-induced growth arrest and IL-6 resistance.

P209

Endomucin: a new endothelial-specific marker in human skin disorders

A. Kuhn[1,2], G. Brachtendorf[2], F. Kurth[2], T. Ruzicka[1], D. Metze[3], D. Vestweber[2,4]

[1]Department of Dermatology, University of Düsseldorf, 40225 Düsseldorf, Deutschland
[2]Institute of Cell Biology, ZMBE, University of Münster, 48149 Münster, Deutschland
[3]Department of Dermatology, University of Münster, 48149 Münster, Deutschland
[4]Max-Planck-Institute of Vascular Biology, 48149 Münster, Deutschland

Endomucin is a classical sialomucin that we recently identified on the surface of mouse endothelial cells and on putative hematopoetic clusters of the dorsal aorta in the embryo. The strong expression of endomucin on high endothelial venules in secondary lymphoid organs and the detection of endomucin as a carrier of the MECA-79 epitope suggested that this novel glycoprotein serves as a ligand for L-selectin. Cloning of human endomucin allowed us to generate monoclonal antibodies (mAbs) against soluble recombinant forms of human endomucin. In the present study, we investigated the expression of this novel molecule in human skin under different conditions. In normal human skin, endomucin was detected by immunoprecipitation and Western blot analysis, and immunohistological analysis of wax-embedded sections revealed that this glycoprotein is specifically expressed on endothelial cells of blood and lymphatic vessels using the mAbs against endomucin. Interestingly, expression on the endothelium of arterioles was either focal or showed weak staining for endomucin compared with control endothelial-specific antibodies. *In situ* hybridization of normal human skin confirmed the expression pattern on the messenger RNA level obtained above. We further analyzed the expression of endomucin in skin biopsy specimens from patients with inflammatory skin diseases such as atopic dermatitis, psoriasis, lichen planus, cutaneous lupus erythematosus, and T cell lymphoma as well as vascular skin tumors such as hemangioma, pyogenic granuloma, angiolipoma, Kaposi's sarcoma, and angiosarcoma. In each case, we found endomucin expressed on the endothelium of the whole vascular tree, concluding that this molecule is a new endothelial-specific marker in the study of human skin disorders.

P210

E-Cadherins Are Involved in Stretch Stimulated Cell Proliferation

F. Kauer[1], S. Kippenberger[1], R. Kaufmann[1], A. Bernd[1]

[1]JW Goethe Universitaet, Dermatology, 60590 Frankfurt/M., Deutschland

Cadherins mediate calcium dependent homophilic cell-cell adhesions and are thought to be involved in cell migration and proliferation with implications to tumor invasion. Adhesion molecules can mediate mechanical impulses and cause mitogen cell reactions in different cell species as demonstrated for beta 1 integrins. In this study we investigated the hypothesis that E-cadherins might be involved in the mechano transduction of human epidermal cells. For this purpose vulva carcinoma-derived A-431 cells were stably transfected with an inducible E-cadherin construct lacking the extracellular domain (kindly provided by K.R. Johnson, Toledo, USA). These Cells were cultivated in flexibl silicon dishes and consecutively stretched twice for 5 min each with a time interval of two hours. After 24 h the incorporation rate of BrdU was determined using a cell proliferation assay (Roche, Mannheim, Germany). Stretching of A-431 cells which contained vectors without the truncated cadherin construct enhanced the BrdU incorporation rate by about 3-5 times. Cells expressing the E-cadherin mutant showed an attenuated response to cell stretch. These results indicate an involvement of E-cadherins in the stretch induced growth stimulation of A-431 cells.

P211

Stretching of Human Keratinocytes results in Co-localization of beta 1-Integrins and EGF-Receptors

Y. Knies[1,2], S. Kippenberger[1], J. Bereiter-Hahn[2], R. Kaufmann[1], A. Bernd[1]

[1]JW Goethe Universitaet, Dermatologie, 60590 Frankfurt/M, Deutschland
[2]JW Goethe Universitaet, Arbeitskreis Kinematische Zellforschung, 60439 Frankfurt/M, Deutschland

Stretching induces activation of MAP-kinases via beta 1-integrins in human keratinocytes [Kippenberger et al. J Invest Dermatol 114:408-412, 2000]. Recent studies suggest a functional and spatial association of integrins and EGF-receptors in cell adhesion. In the present study we tested the hypothesis of a co-localization between beta 1-integrins and EGF-receptors under cell stretch using immunohistochemistry and confocal laser scanning microscopy. We found that in most of the stretched cells beta 1-integrins were assembled in clusters at the basal cell membrane. Such clusters were seen rarely in controls. In stretched cultures of human keratinocytes the frequency of beta 1-integrin-clusters increased about 3 fold compared to controls. Immunohistochemically we found a co-localization of beta 1-integrins and EGF receptors after stretching, which occurred mostly in cluster areas. Concomitantly, the application of cell stretch increased cell adhesion as tested in an usual adhesion assay. This finding suggests the regulation of integrin adhesiveness via an "inside-out signaling", namely the shift from a low affinity to a high affinity state of the fibronectin-receptor. Our results show an enhancement of the adhesion properties of stretched keratinocytes resulting in an increase of focal contacts. The immunhistochemical co-localization of integrins and EGF receptors lead us to assume that beta 1 integrins interact with EGF receptors and might induce a ligand-free activation of the EGF receptors.

P212

Expression of pro-opiomelanocortin-derived peptides in human dermal papilla cells in vitro

M. Eickelmann[1], T. Luger[1], U. Blume-Peytavi[2], M. Böhm[1]

[1]University of Münster, Department of Dermatology and Ludwig Boltzmann Institute for Cell Biology and Immunobiology of the Skin, D-48149 Münster
[2]Univ. Medical Center Charité, Humboldt Univ. of Berlin, Dept. of Dermatology, Center of Experimental and Applied Cutaneous Physiology, D-10117 Berlin

Pro-opiomelanocortin (POMC)-derived peptides such as adrenocorticotropin (ACTH), alpha-melanocyte-stimulating hormone (alpha-MSH) and beta-endorphin (beta-ED) are implicated in a number of biological responses of the hair follicle including pigmentation, hair growth, protection from oxidative stress and regulation of inflammatory responses. However, the exact role of dermal papilla cells (DPC) in this context remains elusive and it is unclear as to whether these cells contribute to the POMC system of the hair follicle. In order to answer this question, we examined if human DPC in culture express POMC, contain POMC processing enzymes and secrete POMC-derived peptides. RT-PCR analysis of DPC derived from scalp from normal volunteers disclosed the presence of transcripts for POMC. Expression of POMC in DPC at the protein level was subsequently confirmed by an anti-ACTH antibody that cross-reacts with POMC. Among the prohormone convertases (PC) that are known to cleave the POMC protein only furin convertase and PC2/7B2 but not PC1 and PACE4 were expressed in DPC. As shown by radioimmune assays and enzyme-linked immunosorbent assays, both DPC lysates and cell culture supernatants contained immunoreactive amounts of ACTH-like peptides as well as beta-ED while alpha-MSH was undetectable. Our findings demonstrate, for the first time, that DPC express and secrete POMC peptides. Moreover, these results provide a basis by which DPC cultures established from scalp from patients with various physiological and pathophysiologic conditions, e. g. early graying or alopecia, can be investigated for their expression pattern of POMC peptides.

P213

Gene expression profiling of human dermal fibroblasts in response to mechanical stimulation.

D. Kessler-Becker[1], A. Seher[1], T. Krieg[1], B. Eckes[1]

[1]Universität Köln, Institut Für Dermatologie, 50931 Köln, Deutschland

Dermal fibroblasts experience variations in mechanical forces in physiological, e.g. body movements, as well as pathological situations, e.g. wound contraction, hypertrophic scars.
We have investigated fibroblast responses to mechanical stimulation using a three-dimensional collagen lattice model system, which reflects certain functional properties of the extracellular matrix in vivo. Fibroblasts were cultured in either mechanically relaxed lattices ("low tension system") or fixed lattices ("stressed system" characterised by increased self generated tensional forces). We compared gene expression profiles, using cDNA microarray technology and Northern analysis, of fibroblasts from stressed and relaxed settings following completion of lattice contraction (20 hours).
Comparison of the transcriptional profile of relaxed and stressed fibroblasts demonstrated that mechanical load regulates a multitude of genes in a differential manner. Tensile forces induced a myofibroblastic cell phenotype characterised by up-regulation of mRNA encoding smooth muscle cell associated cytoskeletal components (e.g. alpha-SMA), proliferation-associated genes, extracellular matrix components and protease inhibitors, growth factors associated with fibrosis (TGF-beta 1, CTGF, MCP-1). By contrast, absence of mechanical tension resulted in concomitant expression of proteases, cell-stress associated gene products and inflammatory mediators. Interestingly, some of these mediators (such as COX-2 and IL-1beta) were regulated in a "biphasic" manner: They were induced at the onset of gel contraction in stressed fibroblasts, while at later time-points, expression was restricted to relaxed cells. Functional blocking of IL-1 action in relaxed lattices revealed that an autocrine IL-1 loop was directly involved in induction of COX-2 and IL-6.
Our results suggest that alterations of the mechanical force balance induce different "activated" fibroblast phenotypes that reflect certain aspects of early wound repair or skin fibrosis.

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Regulation of keratinocyte shape, migration and wound epithelialisation by IGF-1 and EGF dependent signalling pathways.

I. Haase[1], R. Pofahl[1]

[1]University of Cologne, Dept. Dermatology, D-50924 Cologne, Deutschland

Migration of epithelial cells like keratinocytes is a prerequisit for normal wound healing and requires both extension of the plasma membrane and contraction of the cell body. We are investigating growth factor and integrin mediated signaling pathways that regulate keratinocyte migration. In this study we have analysed the cooperation between two different signalling pathways in the regulation of keratinocyte shape, migration and wound epithelialisation in vitro. We have found that signaling mechanisms stimulated by either EGF or IGF-1 and involving ERK and PI-3 kinase activity as well as functions of rho family GTPases induce distinct changes in the cytoskeletal architecture of primary human keratinocytes that affect keratinocyte motility and stimulate wound epithelialisation in an additive manner. Our results suggest that wound epithelialisation is regulated by at least two independent signalling pathways that regulate different components of the cell migration machinery.

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Activated human dermal microvascular endothelial cells (HDMEC) can be separated into subpopulations with distinct expression patterns using the Thy-1 (CD90) as selection marker

A. Saalbach[1], A. Wetzel[2], M. Sticherling[2], U. Haustein[1], U. Anderegg[1]

[1]Sächsische Akademie der Wissenschaften zu Leipzig, D-04103 Leipzig
[2]Universitätshautklinik Leipzig, Experimentelle Dermatologie, 04103 Leipzig, Deutschland

Microvascular EC represent a heterogeneous population differing along blood and lymph vessels, in various organs and fulfilling various functions. Reliable cell markers are the major prerequisite for the detection and analysis of those subpopulations.We have demonstrated that human Thy-1 is an activation marker on a subpopulation of HDMEC. These subpopulations can be separated using antibody coupled microbeads and analysed towards cell biological functions and expression patterns.Microarray analysis was performed to identify differentially expressed genes in Thy-1 positive vs. Thy-1 negative cells.Two RNA pools of Thy-1(+) and Thy-1(-) activated HDMEC each consisting of 5 independent separations were used for 4 hybridi-sations of Affymetrix U95 chips. The hybridisation intensity data were compared from all pools generated among each other (4 comparative analyses). About 100 genes showed expression differences of >2fold after each comparison of Thy-1(+) vs. Thy-1(-) cells. The data were strongly reproducible in Realtime-RT-PCR analysis for highly differently expressed genes (32fold increase) as well as for weakly increased genes (~2fold increase) proving the reliability of the data from the microarray experiment. Among them we found cytokines (IL-7), growth factors (bFGF, glycodelin) and their receptors (VEGFR-2), adhesion molecules (CD209, integrin α9), matrix degrading enzymes (MMP-9, uPA) and gene products involved in inflammation (iNOS, MMR) and immune response (MHCII-DP-βchain). Moreover, these mRNA could be detected in situ in endothelial cells in the human skin. Using Thy-1 as an activation marker of certain HDMEC we are able to identify and characterise new activation associated gene products.

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Increase of hydroxylysine derived collagen cross-links and of lysyl hydroxylase 2 gene expression in systemic sclerosis

J. Brinckmann[1], S. Kim[2], N. Hunzelmann[2]

[1]Universität zu Lübeck, Dermatologie, 23538 Lübeck, Deutschland
[2]Universität Köln, Dermatologie, 50038 Köln, Deutschland

Sclerotic skin diseases are characterized by an increase in hydroxylysine derived collagen cross-links, which are virtually absent in normal skin. An increase in the concentration of hydroxylysylpyridinoline (HP) has been shown for lipodermatosclerosis, keloids and localized scleroderma. In the case of systemic sclerosis, an increase in the urinary excretion of HP has been reported, which has been attributed to a higher turn over of collagen in systemic sclerosis. In this study, we analyzed the cross-link pattern and the expression of collagen modifying enzymes in systemic sclerosis.Cross-link analysis was performed in borohydride-reduced skin specimens after collagenase digestion by amino acid analysis (sclerosis n=9, control n=5). Gene expression of lysyl oxidase, lysyl hydroxylase 1-3, collagen α1(I) and GAPDH were analyzed by RT-PCR (sclerosis n=9, control n=2).In scleroderma, a significant increase in the concentration of hydroxylysine derived cross-links was observed (sclerosis: 0.078 ± 0.034, control 0.029 ± 0.010 mol/mol, $p<0.005$), while the level of lysine derived cross links and the degree of lysyl hydroxylation were unchanged. Furthermore, we observed a marked increase of the gene expression of lysyl hydroxylase 2. The levels of collagen α1(I), lysyl oxidase and lysyl hydroxylase 1 mRNA expression were unchanged, while the expression of lysyl hydroxylase 3 showed only a slight increase. These results demonstrate that systemic sclerosis is accompanied by a change in the pattern of collagen cross-links in skin resulting in an isolated increase of hydroxylysine derived cross-links, which are typical for skeletal tissues. Therefore, the increase of the urinary excretion of HP in systemic scleroosis rather reflects the increased pool of HP in the skin than a suspected higher turn over of collagen. The reason for the altered cross-link pattern seems to be due to an increase in the gene expression of lysyl hydroxylase 2. Our results indicate that this enzyme is responsible for the hydroxylation of lysine residues located in the telopeptide of the collagen molecule and might be a target for a putative therapy of systemic sclerosis.

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Indocyanine green and laser irradiation for selective photothermolysis of blood vessels

P. Babilas[1], R. Engl[1], H. Stockmeier[1], R. Szeimies[1], W. Bäumler[1], C. Abels[1]

[1]Univ. Regensburg, Klinik u. Poliklinik f. Dermatologie, 93042 Regensburg, Deutschland

By careful selection of wavelength, pulse duration, and intensity of a laser blood vessels can be destroyed selectively. So far, a wavelength of 585 nm and a pulse duration of 450 µs are preferred because of selective absorption by endogenous hemoglobin. However, tissue penetration of light at this wavelength is limited. Therefore, to improve the therapeutic outcome of deeper vessels exogenous indocyanine green (ICG) was used absorbing at 805 nm allowing deeper light penetration.
The dorsal skinfold chamber model in hamsters (n = 48) was used for monitoring the vascular effects of ICG (0, 2 or 4 mg/kg b.w.; ICG-Pulsion, Munich, Germany) and diode laser irradiation (λ_{em}= 805 nm; pulse duration 3, 10 or 30 ms; fluence 3.2, 10.6 or 32 J/cm²). Diameters of vessels marked with FITC-dextran (MW 150 000) were measured using intravital fluorescence microscopy prior to, 15 min, 1 h and 24 h following irradiation. At the end of each experiment histology was taken and tissue sections were stained by H&E, NBTC or CD31. A mathematical model calculates the intravascular temperature increase with respect to vessel diameter.
Irradiation with a pulse duration of 30 ms and without ICG did hardly reduce the number of perfused vessels. After irradiation using 2 mg/kg b.w. ICG and 10 ms the number of perfused blood vessels decreased 1 h after irradiation but recovered to baseline at 24 h. Increasing the pulse duration up to 30 ms reduced the number of perfused vessel at 24 h by approx. 30 % at a diameter of 8-10 µm. Using a higher concentration of ICG (4 mg/kg b.w.) and a pulse duration of 30 ms reduced the number of perfused vessels maximally by 53% at a diameter of 7-8 µm. The selective vascular damage was confirmd by histology and immunohistochemistry 24 h after irradiation.
This study shows for the first time the selective destruction of blood vessels following intravenous injection of ICG and subsequent irradiation with a pulsed diode laser.

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The lymphatic system in the dorsal skinfold chamber of the Syrian golden hamster in vivo

H. Brousos[1], V. Schacht[1], D. Berens von Rautenfeld[2], C. Abels[1]

[1]Universität Regensburg, Klinik und Poliklinik für Dermatologie, 93042 Regensburg, Deutschland
[2]Medizinische Hochschule Hannover, Institut für Funktionelle und Angewandte Anatomie, 30625 Hannover, Deutschland

The lymphatic system is of fundamental importance for the regulation of the normal fluid balance in tissues and for the immune response of the organism. Impairment of lymphatic function leads to a number of diseases that are characterized by edema, impaired immunity, or fibrosis. In contrast to the blood circulatory system little is known about the lymphatic system. In particular suitable animal models and specific histological markers are lacking. Therefore, the dorsal skinfold chamber model was investigated regarding the existence of a lymphatic system.To analyze the lymphatic network, we used Syrian golden hamsters (n = 12) fitted with dorsal skinfold chambers. FITC-dextran of different concentrations (5 or 25%) and different molecular weight (4,000, 40,000 or 150,000 Da) was injected intradermally to contrast lymphatic vessels and measure lymphatic velocity. Intravital fluorescence microscopy allowed the quantification of diameter, velocity and branching order.Immediately after intradermal injection of FITC-dextran lymphatics were visible. The diameters of lymphatic vessels (n = 189) ranged from 133 ± 5.4 µm (branching order 1) to 26 ± 4.0 µm (branching order 5). Blood and lymphatic vessels could not clearly be differentiated by H&E stainings. However, demonstration of elastic fibres by orcein staining identified lymphatics and differentiated them from blood capillaries. Furthermore immunhistochemical staining showed that endothelial cells of vessels with an irregularily shaped lumen containing no erythocytes in cross sections showed a weaker signal for CD31 staining as compared to endothelial cells of vessels containing erythrocytes.In this study a lymphatic network was visualized in the dorsal skinfold chamber model of the Syrian golden hamster. This provides a new opportunity to further analyze the function of the lymphatic vascular system under physiological and pathophysiological conditions.

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The endogenous angiogenesis inhibitor thrombospondin 1 is upregulated in acute allergic contact dermatitis in human skin

P. Velasco[1], R. Hügel[1], D. Sicks[1], K. Jahnke[1], J. Brasch[1], E. Christophers[1], J. M. Schröder[1], B. Lange-Asschenfeldt[1]

[1]University of Kiel, Department of Dermatology, 24105 Kiel, Deutschland

In human skin, vascular quiescence is maintained by a tightly controlled balance between proangiogenic growth factors and endogenous angiogenesis inhibitors. Upregulation of angiogenic factors and downregulation of angiogenesis inhibitors is a requisite step in the induction of neovascularization. In cutaneous inflammatory diseases like eczema and psoriasis, increased vascularity and vascular hyperpermeability are characteristic features. Vascular endothelial growth factor A (VEGF-A) and other proangiogenic factors are potently upregulated in inflamed lesions, conversely, the endogenous angiogenesis inhibitor thrombospondin 2 (TSP) –2 was recently found to play an important role in the control of an experimental delayed-type hypersensitivity reaction in the skin of mice. In order to investigate the biologic role of angiogenesis in human cutaneous inflammation, we quantified the vascularity (number, size and density) in acute allergic contact dermatitis and normal skin using computer-assisted morphometric image analysis of collagen-IV and CD31(PECAM-1)-stained frozen sections. Furthermore, we compared the levels of TSP-1 expression in inflamed lesions and healthy skin from the same donor. We report that vessel density in the inflamed tissue was already increased 72 hours after the onset of an inflammation (our earliest timepoint) despite evident overexpression of an endogenous angiogenesis inhibitor. We also found a correlation between the severity of the inflammation and the level of TSP-1 overexpression. Surprisingly in severely inflamed tissues, we observed diffused TSP-1 expression predominantly in the upper papillary dermis with a gradient into the deeper dermis. Moreover, we report localized expression of TSP-1 in the blood vessels of these lesions. These results reveal modulation of TSP-1 expression in acute allergic contact dermatitis in human skin, suggesting a paradox between the known antiangiogenic activity of TSP-1 and its proinflammatory function. Perhaps during early stages of inflammation, the proinflammatory effects of TSP-1 are outweighing its ability to directly inhibit endothelial cell proliferation and migration. Additional investigations will elucidate this question.

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Emerging phenotypes of mice lacking integrin α2β1

O. Holtkoetter[1], B. Nieswandt[2], K. Strick[1], K. Elias[1], M. Aumailley[3], N. Smyth[3], T. Krieg[1], B. Eckes[1]

[1]University of Cologne, Dermatology, 50931 Köln, Deutschland
[2]Rudolf-Virchow Center, 97078 Wuerzburg, Deutschland
[3]University of Cologne, Biochemistry II, 50931 Köln, Deutschland

Cell - extracellular matrix interactions play vital roles in development and tissue homeostasis, and are mediated predominantly by receptors of the integrin family. The collagen receptor α2β1 is ubiquitously expressed and, based on *in vitro* data, required for cell migration, matrix contraction, tissue remodeling and hemostasis. In order to analyze the function of α2β1 *in vivo*, we generated mice that lack the α2 subunit completely and mice in which the deficiency is restricted to epidermal keratinocytes. To our great surprise, mice deficient in α2β1 develop apparently normally and reproduce, indicating that no major developmental defects arise from α2-deficiency. The skin of these animals is normal at gross morphological inspection. However, functional analysis of fibroblasts and keratinocytes cultured from the skin of α2-/- animals revealed impairment in attachment, migration, contractile capacity as well as cytoskeletal abnormalities. We postulate that these functions will result in impaired biological responses to stress at the tissue or organismal level.Further, α2β1 was postulated as the major collagen receptor on platelets, mediating platelet adhesion to collagen and required for the formation of a hemostatic plug following injury. Indeed, subtle differences in adhesion and collagen-induced aggregation by α2-deficient platelets were observed. However, bleeding times and platelet counts were normal in α2-/-, but blocking the collagen receptor GPVI on α2-/- platelets completely abrogated adhesion to collagen fibrils. The α2-/- mouse provided the first unequivocal evidence for the hierarchy of GPVI beinf required for and α2β1 playing a supportive rather than essential role in platelet-collagen interactions.

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Hyperandrogenism in male acne patients

M. Placzek[1], B. Arnold[1], H. Schmidt[2], E. Albert[2], E. Keller[2], G. Plewig[1], K. Degitz[1]

[1]Ludwig-Maximilians-Universität München, Klinik und Poliklinik für Dermatologie und Allergologie, 80337 München, Deutschland
[2]Ludwig-Maximilians-Universität München, Kinderklinik und Poliklinik im Dr. von Haunerschen Kinderspital, 80337 München, Deutschland

Androgen excess may provoke or aggravate acne by inducing seborrhea. In women androgen disorders are frequently suspected when acne is accompanied by hirsutism or menstrual irregularities. In men, however, acne may be the only symptom of androgen excess. In order to investigate the frequency and nature of androgen disorders in male acne patients, we have examined 106 consecutive male out-patients in whom the diagnosis mild to severe acne was made (mean age 24.0 years, age range 14.4 ? 43.8 years). Assessed parameters included morning serum values of testosterone, dehydroepiandrosterone-sulfate (DHEA-S), androstenedione, and 17-hydroxyprogesterone (17-OHP); an ACTH stimulation test; and genetic screening for 9 frequent mutations in the 21-hydroxylase (*CYP21*) gene associated with congenital adrenal hyperplasia (adrenogenital syndrome). We detected one or more increased baseline androgen values in 74/106 patients (testosterone 0/106, DHEA-S 17/106, androstenedione 11/106, and 17-OHP 65/106). The ACTH stimulation test revealed abnormal 17-OHP induction in 17/106 patients. In 10/106 patients, mutations in the 21-hydroxylase gene were detected. The results suggest that 1) in men acne is not infrequently accompanied by hyperandrogenism and 2) congenital adrenal hyperplasia may not be the only pathogenetic mechanism underlying hyperandrogenism.

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Hair cycle dependent expression of estrogen receptor α and β in murine skin

U. G. Ohnemus[1], M. Nakamura[2], S. Liotiri[1], F. Conrad[3], L. Mecklenburg[4], R. Paus[1]

[1]Universitaetskrankenhaus Hamburg Eppendorf (Universitaet Hamburg), Abteilung fuer Experimentelle Dermatologie, 20246 Hamburg, Deutschland
[2]Dep. Of Dermatology, Postgraduate School of Medicine, 606-8507 Kyoto, Japan
[3]Institut fuer Anatomie, Tieraerztliche Hochschule, 30625 Hannover, Deutschland
[4]Institut fuer Pathologie, Tieraerztliche Hochschule, 30625 Hannover, Deutschland

Our knowledge of estrogen function and target cells in hair biology is still very limited. The two recognized estrogen receptors, ERα and ERβ, display different immunolocalization profiles in human skin, suggesting different functional roles in cutaneous biology. Previously it has been reported that ERα is expressed only in the DP and ORS of telogen and early anagen mouse hair follicles, while ERβ was undetectable. We, therefore, studied ERα and ERβ expression in hair follicles of C57BL/6 mice throughout the murine hair cycle by IHC and RT-PCR. Nuclear IR was detected for both ERα and ERβ throughout all investigated hair cycle stages (telogen, anagen VI, catagen). ERα IR had its peak in telogen follicles within the DP and the SG while the IRS and ORS showed weaker IR. In anagen VI, we detected ERα IR in the ORS and the DP, while in early catagen it was restricted to the DP and the secondary hair germ. In anagen VI follicles ERβ was weakly positive in hair matrix and ORS, while in catagen and telogen follicles ERβ was expressed in the DP, IRS, ORS and the SG. By semiquantitative RT-PCR, we detected ERα mRNA in telogen, anagen V and VI and late catagen skin extracts, with peak transcript levels in telogen skin. Taken together, our data show that ERα and β are not only expressed during telogen and early anagen but also that ERβ is indeed expressed in murine skin and suggest that endogenous estrogens play an important role in murine hair cycle control.

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Human hair follicle organ culture as a screening tool for "hair drug" discovery: a reconsideration

A. Mescalchin[1], A. Zambon Bertoja[1], P. Pertile[1], R. Paus[2]

[1]Cutech Srl, 35129 PADOVA, Italien
[2]University Hospital Eppendorf, Dept. of Dermatology, 20246 Hamburg, Deutschland

The organ culture of human hair follicles (Philpott *et al.*, J Cell Sci 1990, 97 pt 3: 463-71) currently is the only acceptable and available *in vitro*-method for predicting how any given test agent might affect human hair growth *in vivo*. Scalp or facial hair follicles in anagen VI are truncated below the dermis, and are cultured up to 10 days, showing growth of a pigmented hair shaft at about the *in vivo* rate. Hair shaft elongation is measured as an indicator of hair growth inhibition or stimulation by the test agent. Here, we are reconsidering the use and the limitations of this seminal hair research tool and suggest modifications for its optimisation. This assay is best-suited to test hair growth-inhibitory candidate hair drugs, since anagen VI hair bulbs show already maximal hair shaft production activity and are therefore very susceptible to inhibitory compounds. Also, this assay system imitates systemic, but not topical drug administration in man, and the absence of large portions of the pilosebaceous apparatus (incl. the stem cell-bearing bulge and the sebaceous gland) limits the physiological relevance of this model. Finally, the interindividual and interfollicular variations in test drug response are very large, hindering its pharmacological usefulness. We have employed changes e.g. in the culture conditions, in how hair follicles are selected for organ culture and how hair shaft elongation is recorded/calculated. The read-out parameters were enlarged and standardized, including quantitative immunohistomorphometry for proliferation/apoptosis of hair matrix keratinocytes after 2 and 9 days (TUNEL/Ki67/DAPI triple stain), quantitative hair cycle histomorphometry of spontaneous or TGFβ-induced catagen development, and analysis of pigmentary phenomena (melanin quantity/distribution, number of NKI/beteb+ melanocytes). These modifications greatly enhance its usefulness as a screening tool for candidate "hair drugs", if one fully recognizes the limitations of this assay.

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DOES A HORMONE REPLACEMENT THERAPY INFLUENCE SKIN AGEING?

P. SATOR[1,2], J. B. SCHMIDT[1], M. O. SATOR[3], J. C. HUBER[3], H. HOENIGSMANN[1]

[1]General Hospital, Department of Special and Environmental Dermatology/University of Vienna, 1090 Vienna, Oesterreich
[2]Hospital Lainz, Department of Dermatology and Venereology, A-1130 Vienna
[3]General Hospital, Department of Obstetrics and Gynaecology/University of Vienna, 1090 Vienna, Oesterreich

Objectives: We studied the effect of hormonal treatment on skin ageing in menopausal women.
Methods: 24 patients (45-68 yrs, mean age 54.9 yrs) without hormone treatment for at least six months were included. Patients were assigned to three therapy groups: 1. Oestrogen only (Estraderm TTS® 50) (n=6), 2. Transdermal oestrogen and progesterone (Estraderm TTS® 50 and progesterone vaginal suppository 0.4 mg) (n=7) and 3. Oral oestrogen and progesterone (Progynova® 2 mg and progesterone vaginal suppository 0.4 mg) (n=8). One group without therapy was included as a control group (n=3). Treatment was continued for 6 months. Due to the discontinuation of treatment by one patient in group 2 and by two patients in group 3, six patients remained in each group with HRT. The following skin parameters were measured at monthly intervals during treatment: skin surface lipids, epidermal skin hydration, skin elasticity and skin thickness. Concomitant clinical evaluation included a subjective clinical evaluation form, a patient questionnaire and laboratory tests for oestradiol, progesterone and follicle stimulating hormone.
Results: Mean levels of epidermal skin moisture, elasticity and skin thickness were improved at the end of treatment based on both subjective and objective evaluation in patients with HRT. Skin surface lipids were increased during combined HRT, which may reflect stimulatory effects of the progestagen component on sebaceous gland activity, while oestrogen alone has a sebum-suppressive action. In the HRT groups the questionnaire for climacteric complaints demonstrated significant improvements while laboratory tests showed increases in oestradiol and progesterone and decreases in FSH.
Conclusions: HRT with the mentioned regimes significantly improved parameters of skin ageing.

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Calcium-fluorescence lifetime imaging in ex-vivo skin

M. J. Behne[1], N. P. Barry[2], E. Gratton[2], T. M. Mauro[1]

[1]University of California San Francisco, Dermatology Department (190), 94121 San Francisco, USA
[2]University of Illinois Urbana-Champaign, Department of Physics, Laboratory for Fluorescence Dynamics, 61801 Urbana, USA

Calcium controls several key events in keratinocytes and epidermis: differentiation, signaling and cell adhesion. To date, two genodermatoses have been identified with an underlying defect of Ca2+ homeostasis, namely Darier Disease, due to mutations in the ER-calcium ATP'ase, ATP2A2, and Hailey-Hailey Disease, due to mutations in the Golgi-calcium ATP'ase, ATP2C1. Both diseases lead to an intracellular Ca2+ imbalance, with the ultimate effect of impaired cell adhesion. Additionally, disturbed Ca2+ signaling may be involved in other pathogenetic processes, such as Psoriasis. Nevertheless, the tissue- and cellular Ca2+ concentrations in these diseases are only partially known. Determinations of the Ca2+ gradient in intact skin have been made by electron microscopy, using Ca2+-precipitation histochemistry, or by PIXE. Both techniques are limited in that they can determine Ca2+ in only very small sample volumes, at or below light microscopic resolution levels, require fixed tissue and a chemical precipitation, or determine only total Calcium, irrespective of ionization or binding. So far, neither cellular and/or subcellular localization can be determined through these approaches. Previously, these methods have been employed for numerous investigations into the epidermal Ca2+ gradient. Nevertheless, a method that allows to measure and localize Ca2+ in intact and unfixed tissue has been lacking. In cells, fluorescent dyes have been used extensively for ratiometric measurements of static and dynamic Ca2+ concentrations, and in recent publications, we described Fluorescence Lifetime Imaging Microscopy to assess pH in intact epidermis samples. Here we report about the first experiments to measure and visualize Ca2+ in ex-vivo biopsies of unfixed epidermis, exploiting FLIM to measure Ca2+ concentrations. Our initial experiments show overall increasing Ca2+ concentration from SB to SG, with very low levels in the SC, confirming PIXE and Ca2+-precipitation results. Within the SG, highest Ca2+ can be found in a granular distribution intracellularly. We plan to use this new technique in ex-vivo samples to assess dynamic changes of Calcium and to gain new insights into various pathologic situations.

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Stress and hair follicle melanocyte apoptosis: a reason for premature graying?

E. Peters[1], K. Spatz[1], E. Hagen[1], R. Overall[1], P. Arck[1]

[1]Psychoneuroimmunology, Biomedical Research Center, Charité, Humboldt University, 10099 Berlin, Deutschland

Now and again, premature greying is mentioned in the context of stressful life-events, e.g. anecdote reports, that Marie-Antoinette lost all hair colour immediately after sentenced to death. The pigment-production by hair follicle melanocytes produces constant oxidative stress for the cells. Stress-induced cutaneous inflammation can add to this and may kick melanocyte-stress regulatory mechanisms of balance, ultimately leading to premature death of melanocytes and white regrowth of hair e.g. in alopecia areata. To investigate the effects of major stress-mediators such as the neuropeptide substance P (SP) on the follicular pigmentary-unit, we employed an experimental approach using C57BL/6 mice which grow a black fur and have been extensively used for hair- and stress-research. Mice had all their back skin hair follicles removed by depilation to induce homogeneous hair growth (anagen). After onset of pigment-production in the growing hair follicles, mice received a bolus injection of SP. Skin was harvested for immunohistochemistry and melanocyte apoptosis was assessed by double staining of a melanocyte marker (tyrosinase related peptide 1, courtesy of Vincent Hearing) and TUNEL-labelling (apoptosis). SP treatment resulted in melanocyte-apoptosis in the developing pigmentary-unit of early anagen hair follicles. In addition, the number of melanocytes in the outer root sheath increased after injection of SP. Our findings suggest, that stress-mediators can cause premature death of pigment-producing hair bulb melanocytes in early anagen, when the pigmentary-unit is freshly formed and starting to function. At the same time, stress-mediators may cause compensatory activation of the melanocyte stem-cell reservoir in the outer root sheath. These findings open up a new field for the investigation and development of therapeutic approaches aiming at the reduction of stress-effects on aging processes such as premature graying.

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Evidence for a Proinflammatory Role of Proteinase-activated Receptor-2 during Cutaneous Inflammation in vivo.

M. Steinhoff[1], S. Seeliger[1], C. Derian[3], R. Nawroth[2], C. Sunderkötter[1], D. Metze[1], D. Vestweber[2], P. Andrade-Gordon[3], T. A. Luger[1].

[1]University of Muenster, Dep.of Dermatology, 48149 Muenster, Deutschland,
[2]Dept. of Cell Biology, Center for Molecular Biology of Inflammation (ZMBE), University of Münster, 48149 Muenster, Germany;
[3]R.W. Johnson Pharmaceutical Res Institute, Spring House, USA

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

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Differentiation specific localization of acetylcholine receptors in human skin: implications for skin physiology

H. Kurzen[1], H. Berger[1], .C. Jäger[1], W.g Hartschuh[1], H. Näher[1]

[1]Universität Heidelberg, Hautklinik, 69115 Heidelberg, Deutschland

Extraneuronally, acetylcholine (Ach) is synthesized, stored and secreted in the tegumental cells covering the inner and outer surfaces of the body. It acts via nicotinic (nAch-R) and muscarinic (mAch-R) receptors in a paracrine and autocrine fashion influencing keratinocyte proliferation and differentiation. In order to study the role of the cholinergic system in the skin under physiologic and pathologic circumstances, we examined the expression nicotinic and muscarinic acetylcholine receptors in human skin with own and commercially available antisera. Using immunohistochemistry, we found interindividually and intraindividually highly variable staining patterns. In some parts of the epidermis, especially periinfundibularly, there was a strong staining of the epidermal basal layer for the α3-, α5-, β2- und β4-subunit antisera. In addition to the localization in the basal layer, we found a cytoplasmic colocalization of the α3, α5 and β2 chains in a single cell layer of the stratum granulosum. The alpha 7 and 9 subunits, which form homooligomeric receptors, were predominantly expressed in the suprabasal layers. Like in other extraneuronal systems, the m4 and m5 isoforms of the muscarinic receptors were broadly distributed, while the m1 isoform was restricted to the suprabasal cells of the upper spinous and granular layers. We could confirm the presence of alpha 3, 5, 7 9, beta 1, 2 and 4 nAch-R and the m1-m5 mAch-R in human skin on mRNA-level by PCR and in situ hybridization. Unexpectedly we found the beta 1 chain in upper stratum spinosum and in the stratum granulosum. We conclude, that proliferating basal cells of the epidermis express two types of Ach-R: the heterooligomers of the nAch-R alpha 3* type and the mAch-R m3, weakly also m4 and m5. Upon differentiation keratinocytes of the spinous and granular layer downregulate the alpha 3* and the m3 receptors and upregulate alpha 7, alpha 9, beta 1 and m1. Our data imply a crucial role of the cholinergic system in various processes of skin physiology, especially terminal differentiation.