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Contents

V01

Unsettling news: Indigestion medications promote type I allergy to food proteins

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Gastric digestion is mediated by pepsins which get activated upon exposure to low pH in stomach fluid. Food allergens that sensitize via the gastrointestinal tract are considered to be resistant to peptic digestion. Consequently, the allergenic potency of food proteins is today evaluated by in vitro assays using simulated gastric fluid (SGF). Our hypothesis was that a dietary protein may attain sensitizing capacity when peptic digestion is disturbed. Indeed, slight elevation of the pH in in vitro assays hindered pepsin activation and thus protein digestion. In a previous experimental model, we simulated hypoacidity in BALB/c mice by simply treating them with anti-ulcer drugs, such as Sucralfate, Omeprazole or H2-receptor blockers. In this situation, feedings of normally digestion-labile proteins resulted in a food-allergic phenotype, with specific IgE and IgG1, positive type I skin reactivity and eosinophilia of the gastric mucosa. Consequently, in the present study we examined whether 152 patients who were acid suppressed for indigestion complaints developed food hypersensitivities, and compared them to 50 untreated controls. Interestingly, 3 months of anti-ulcer therapy resulted in an increase in food specific IgE in 25% of all treated patients (n=152), which was either due to a boost of pre-existing food specific IgE in 10%, or de novo IgE formation in 15%. Thus the risk to develop IgE during anti-ulcer therapy is 10.5 (95% confidence interval: 1.44 - 76.48; p=0.0203). Five months after discontinuation of therapy, still 6% had food-specific IgE accompanied by positive skin reactions. The changes in IgE were paralleled by a highly significant elevation of the Th2 marker ST2 in sera of patients (p=0.016). Our data strongly suggest that anti-ulcer drugs impair the gate-keeping function of the stomach for food allergens, turning harmless proteins into allergens. This situation is ignored by food safety tests using SGF. As estimated 10% of the adults do regularly consume anti-ulcer drugs and sales numbers do steadily increase, we suggest our findings to represent unsettling news.

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V02

Dendritic cells phagocytose L. major parasites via FcγRI (CD64) and FcγRIII (CD16)

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In earlier studies we have shown that parasite uptake by dendritic cells (DC) is dependent on Fcγ-receptors (FcγR). Leishmania-infected DC release IL-12 and exhibit MHC class I and II-dependent presentation of L. major-antigens, whereas in macrophages phagocytosis of the parasite via CR3 leads to inhibition of IL-12 and class II-restricted antigen presentation. In addition, we showed that infected DC are able to effectively vaccinate against progressive disease. We now attempted to identify the exact receptor for parasite uptake by DC. All 3 known FcγR bind IgG: Complexed IgG1 binds preferentially to FcγRIII and IgG2a/b complexes bind with higher affinity to FcγRI. FcγRII is an effective receptor for endocytosis of soluble complexes. First, we analyzed the isotypes of anti-Leishmania-Ab bound to the surfaces of L. major. We detected both IgG1 and IgG2a/b on the parasite surface. Functional studies revealed that total IgG (enriched from serum of infected mice using protein G-columns) mediated phagocytosis, whereas IgM and IgE - although detected on parasite surfaces - were not involved. Next, we generated bone marrow-derived DC from single and double receptor-deficient mice as well as Fcγ common chain knockout mice to investigate the exact role of each of these receptors in phagocytosis assays with L. major. As expected, ~70% fewer parasites were parasitized by DC generated from Fcγ-/- compared to wild types or by FcγRI-/- DC additionally treated with inhibitory anti-FcγRII/III mAb clone 2.4G2. Parasite uptake was not altered in DC from FcγRI, FcγRII or FcγRIII-/- mice, whereas significant inhibition was found when FcγRI/III double deficient mice were used. In vivo, enhanced recruitment and infection of DC initiated by inoculation of IgG-opsonized parasites led to enhanced Th1 immunity and rapid resolution of cutaneous lesions compared to infections with non-IgG-treated parasites. In summary, FcγRI and -III each play a critical role in the phagocytosis of L. major by DC and these receptors can efficiently compensate for one another. In addition, IgG-mediated phagocytosis of L. major by DC appears to be responsible for the rapid development of protective anti-parasite immunity.

V03

PREDICTION OF DISEASE PROGRESSION IN MELANOMA BY SERUM PROTEOMIC PROFILING

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Purpose: Currently known serum biomarkers do not predict clinical outcome in melanoma. S100-beta is widely established as a reliable prognostic indicator in patients with advanced metastatic disease but is of limited predictive value in tumour-free patients. This study was aimed to determine whether molecular profiling of the serum proteome could discriminate between early and late stage melanoma and predict disease progression.
Patients and Methods: 205 serum samples from 101 early stage (AJCC I) and 104 advanced stage (AJCC IV) melanoma patients were analysed by matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (ToF) mass spectrometry utilizing protein chip technology and artificial neural networks (ANN). Serum samples from 55 additional patients after complete dissection of regional lymph node metastases (AJCC III) with 28/55 patients relapsing within the first year of follow-up were analysed in an attempt to predict disease recurrence. Serum S100-beta was measured using a sandwich immunoluminometric assay.
Results: Analysis of 205 stage I/IV serum samples, utilizing a training set of 94/205 and a test set of 15/205 samples for 32 different ANN algorithms, revealed correct stage assignment in 84/96 (88%) of a blind set of 96/205 serum samples. 44/55 (80%) stage III serum samples could be correctly assigned as progressors or non-progressors using boot-strap statistical validation. 23/28 (82%) stage III progressors were correctly identified by MALDI-ToF combined with ANN, whereas only 6/28 (21%) could be detected by S100-beta.
Conclusion: Further validation of these findings may enable proteomic profiling to become a valuable tool for identifying high-risk melanoma patients eligible for adjuvant therapeutic interventions.

V04

Siehe P251

V05

An important role of CD80/CD86-CTLA-4 signaling during photocarcinogenesis

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The development of skin cancer seems to be controlled by the immune system and B7 mediated co-stimulation (CD80/CD86-CD28/CTLA-4 pathway) plays a fundamental role in UV-induced regulation of immunity. To investigate CTLA-4 signaling during photocarcinogenesis chronically UV-exposed mice were treated with a blocking anti-CTLA-4 antibody. Anti-CTLA-4 treated mice developed significantly fewer UV-induced skin tumors and additionally, anti-CTLA-4 treatment induced long lasting protective antitumoral immunity. Since CTLA-4 binds to CD80 and CD86 we used mice either deficient for CD80, CD86 or both for photocarcinogenesis studies and interestingly, double deficient mice showed a significantly reduced skin tumor development. Growth of UV tumors appears to be controlled by UV-induced suppressor T cells. Indeed, CD80/CD86 deficient mice had dramatically reduced numbers of CD4+CD25+ suppressor T cells. Since UV-induced CD4+CD25+ suppressor T cells express CTLA-4 as evidenced by flow cytometry we investigated if anti-CTLA-4 treatment would influence the number and function of UV-induced CD4+CD25+ suppressor T cells. In vitro blockade of the CTLA-4 pathway abrogated the suppressor activity of UV-induced CD4+CD25+ T cells completely suggesting that reduced photocarcinogenesis might be due to decreased numbers or function of suppressor T cells. To elucidate the role of UV-induced CD4+CD25+ suppressor T cells and CTLA-4 signaling for antitumoral immunity in more detail we transferred UV-induced regressor tumors into CD80/CD86 deficient recipients and injected UV-induced CD4+CD25+ suppressor T cells as well as anti-CTLA-4. As expected, tumors were rejected in wildtype and B7-1/2 double deficient mice but interestingly, tumor growth was dramatically increased when mice were injected with UV-induced CD4+CD25+ T cells. Moreover, when recipient mice were additionally treated with anti-CTLA-4 to block the function of UV-induced suppressor T cells all tumors were rejected. Together, these data indicate that interfering with CD80/CD86-CTLA-4 signaling results in immune protection against the development of UV-induced skin tumors. Furthermore, the CD80/CD86-CTLA-4 pathway is critically involved in the peripheral homeostasis and function of UV-induced suppressor T cells.

V06

Activated CD4+CD25+ T cells suppress antigen specific CD4+ and CD8+ T cells but induce a suppressive phenotype only in CD4+ T cells

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CD4+CD25+ regulatory T cells are increasingly recognized as central players in the regulation of immune responses. In vitro studies have mostly employed allogeneic or polyclonal responses to monitor suppression. Little is known about the ability of CD4+CD25+ regulatory T cells to suppress antigen specific immune responses in humans. It has been previously shown, that CD4+CD25+ regulatory T cells anergize CD4+ T cells and turn them into suppressor T cells.
In the present study we demonstrate for the first time in humans that CD4+CD25+ T cells are able to inhibit the proliferation and cytokine production of antigen specific CD4+ and CD8+ T cells. This suppression only occurs when CD4+CD25+ T cells are preactivated. Furthermore, we could demonstrate that CD4+ T cell clones stop secreting IFN-g, start to produce IL-10 and TGF-b after co-culture with preactivated CD4+CD25+ T cells and become suppressive themselves. Surprisingly preactivated CD4+CD25+ T cells affect CD8+ T cells differently, leading to reduced proliferation and reduced production of IFN-g. This effect is sustained and cannot be reverted by exogenous IL-2. Yet CD8+ T cells, unlike CD4+ T cells do not start to produce immunoregulatory cytokines and do not become suppressive after co-culture with CD4+CD25+ T cells.

V07

G Protein Beta 3 Subunit Gene TT Genotype is A Novel Genetic Risk Factor For Accelerated Progression to AIDS

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The natural course of progression to AIDS is significantly influenced by genetic host factors. Previous studies have mainly focused on genetic polymorphisms in chemokine receptors which constitute the main entry route for NSI and SI isolates. However, signal transduction via G protein-coupled receptors was shown to enhance the subunit of heterotrimeric G proteins is highly predictive of enhanced signaling via Gi proteins in man. We hypothesized that increased G protein signaling in 825T allele carriers could be associated with an accelerated progression to AIDS. We completed a retrospective cohort study on 523 living seroprevalent patients (39 females, 484 males) with sexually acquired HIV-1 infection at specialized centers located in Bonn, Essen and Frankfurt (95,6% Caucasian, 3,8% African, 0,6% Asian). End points studied were i. time to AIDS acccording to CDC 1993 definition, ii. time to CD4 cell count <200/μl, and iii. time to highest viral load as determined by standard RT-PCR technique. genotyping for the GNB3 C825T polymorphism was conducted. Kaplan-Meier curves were prepared to describe event-free survival until progression to AIDS and maximum virus load. Age- and sex-adjusted relative hazards were calculated based on the Cox proportional hazards models. Continuous variables were compared using the Kruskal-Wallis. "RH" denotes relative hazard, "CI" indicates 95% confidence interval limits. GNB3 825T allele frequency was 355,3% (64TT, 239 TC, 217 CC) and genotype distribution was compatible with Hardy Weinberg equilibrium. While progression to AIDS was almost identical in individuals with TC and CC genotypes, a significantly enhanced progression was associated with the TT genotype. RH for progression to AIDS was 1,93 (CI: 1,43-2,61; p<0,0001;TT versus TC/CC) and RH for CD4 T cell decline to below 200/μl was 1,83 (CI: 1,34-2,50; p<0,0001). Individuals with TT genotype attained their highest virus load earlier than those with TC/CC genotypes (RH 1,49; CI: 1,14-1,93; p=0,003). Mean AIDS-free survival was 2,5, 4,1 and 3,9 years for TT, TC and CC genotype, respectively (p==0,0155).
We conclude that genetically fixed enhanced G protein activation is a risk factor for progression to AIDS. The GNB3 TT genotype is associated with a significant recessive disease-accelerating effect

V08

Intranasal instillation of antigen loaded DC induces rapid and long lasting antigen specific immune response and non responsiveness to aerosol challenge

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We evaluate the impact of a single intranasal sensitization with antigen loaded DC subsets on humoral immune responses, T cell cytokine production and allergen induced broncho-alveolar lavage (BAL) cellular infiltrate. BALB/c bone marrow derived DC were generated under the agis of Flt-3 ligand (FL-DC) or GM-CSF (GM-DC) containing plasmacytoid DC and myeloid DC, respectively. DC were loaded with OVA (1mg/ml) for the final 12 hrs in presence or absence of CpG-ODN (1μM). OVA loaded DC (106/25l) were administered by single intranasal installation and OVA specific humoral response was determined. BAL analysis was performed 24hrs after 2x OVA aerosol challenge (d166 and 167). IL-4, IL-5 and IFN-γ release of MACS sorted CD4 splenocytes (d168) was analyzed by ELISA. Mice sensitized with OVA loaded DC developed a rapid (earliest time point d14) and long lasting (>130d) Ab response. CpG activated FL-DC induced a solid IgG2a response (Th1 dominated) but failed to induce IgE or IgG1. In contrast, GM-DC induced a mixed profile (IgG2a, IgG1 and IgE). CpG activation led to a shift with increased IgG2a and decreased IgG1 and IgE. Despite elevated OVA-specific IgE/IgG1 levels, mice sensitized by OVA loaded DC when challenged with OVA aerosol did not develop allergic inflammation of the lung (while control mice sensitized i.p. with OVA/alum did). Splenic CD4 T cells obtained from DC-sensitized mice 24 hrs after aerosol challenge demonstrated high level of spontaneous cytokine production, suggesting a long lasting memory effect also on the cellular level. Consistent with the humoral response, CD4 cells from GM-DC sensitized mice produced significantly more IL-4 and IL-5 spontaneously and upon OVA restimulation as cells obtained from FL-DC sensitized mice. In conclusion, a single intranasal administration of antigen loaded DC induces a rapid and long lasting humoral and cellular immune response. Despite high levels of antigen specific IgE and IgG1, animals are non responsive to aerosolized antigen challenge, suggesting that intranasal administration of antigen loaded DC may convey protection against inflammatory responses to harmless airborne allergen.

V09

SKIN-HET-CAM : An in-vivo absorption model, using perfused, living human skin

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There are several models to describe the absorption of drugs into the different layers of human skin. Standardized and well-known methods are needed to apply the results to the conditions of the human organism and to economize in vivo experiments. The objective is to develop an absorption model for studying topical formulations, in order to get reproduceable results by using the actual biological system of skin.
Nowadays the number of animal tests could be reduced by using the chorioallantoic membrane of chicken eggs. As an intermediate stage between isolated cells and animal experiments, the CAM model system is a borderline system between in-vivo and in-vitro models and does not raise ethical or legal questions or violate animal protection laws. The HET-CAM-model (Hen#s Egg Test-Chorio Allantois Membrane model) is an alternative in-vitro method to the commonly used "Draize Eye Test". The CAM also provides an excellent natural substrate for all kinds of tumor cells. Chicken embryos are naturally immunodeficient hosts that can accept transplantation of various xenogenic tissues. The modification of this established model leads to an in-vivo model system for the biopharmaceutical evaluation of topical formulations on human skin.
Adult human full skin was grafted onto the chorioallantoic membrane of 5-day chicken embryos (SKIN-HET-CAM). The influence of vascular endothelial growth factor (VEGF), dimensions of the grafts and the duration of incubation of the rate of successfully transplantation were examined. We also compared the new "Skin-HET-CAM-model" with an established absorption model - the "diffusion chamber by Franz". There are a few differences in reference to the extent and speed of drug penetration into the skin and the extent of the systemic bioavailability.

V10

Ephrin-B2 signaling and integrin-β1 interaction is linked to Lubrol-RAFT compartmentalization in B16 melanoma cells

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Ephrin-B2 cell-cell signaling has recently been reported to enhance integrin-mediated ECM-attachment and contribute to a migratory phenotype in B16 melanoma (MM) cells. For functional ephrin-B, special cholesterol-rich microdomains (RAFTs) are essential to provide the necessary proximity for interacting proteins in the membrane-compartment. However, it is unknown if transformation and tumor progression in the melanocytic system are linked to changes of RAFT-composition and mobility.
To demonstrate that the membrane-bound signaling molecule ephrin-B2 is targeted to such microdomains in full-length ephrin-B2 transfected B16 mouse MM cells, RAFT membrane fractions were prepared in sucrose gradients and immunoblotted applying anti-CD29 (integrin-β1) and anti-ephrin-B2 antibody: Lubrol-WX insoluble RAFTs showed a considerable amount of ephrin-B2 in the immunoblot, in contrast to Triton-X 100 RAFTs. Integrin-β1, however, was detectable in both types of RAFTs. Therefore, in the transfected B16 model cell line a functional interaction between ephrin-B2 and integrin-β1 might be assumed in close proximity within clusters of Lubrol-RAFTs. Cyclodextrin (CD) treatment for cholesterol depletion resulted in disruption of the RAFT-composition and loss of ephrin-B2-detectability in the detergent-insoluble membrane fraction in the immunoblot; RAFT-compartmentalization of integrin-β1 was less susceptible. Cholesterol-depletion and disruption of RAFTs by non-toxic CD-dosis prior to cell seeding was highly effective in terms of blocking the integrin-mediated ECM-attachment. These findings support the concept that ephrin-B2 localization to RAFTs stimulates integrin-mediated attachment. Consistent with a close functional interaction of integrin-β1 and ephrin-B2, confocal fluorescence microscopy of integrin-β1 and ephrin-B2 demonstrated a partly overlapping subcellular distribution of both molecules within the lamellipodial protrusions of the B16-transfectants.
Conclusively, the preference of ephrin-B2 for Lubrol-WX RAFTs and its partial colocalisation with integrin-β1 are novel in the B16 MM model system. Further investigation of the mechanisms of RAFT-compartmentalization and trafficking of ephrins and interacting integrins could become a new field for experimental molecular targeting of metastatic MM.

V11

Inhibition of epidermal acetylcholine receptors severely disrupts epidermal homeostasis

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Increasing evidence suggests an important role of the extraneuronal cholinergic system in skin physiology. The aim of the present study was to analyse the influence of cholinergic (nicotine, carbachole, muscarine) and anticholinergic drugs (mecamylamine, atropine, strychnine) on epidermal physiology using organotypic cocultures (OTC) as an in vitro skin equivalent system. Immunofluorescence analysis was performed using a panel of antibodies against Mib-1, various differentiation markers and adhesion molecules. Nile red was applied for visualization of lipids. Blocking of all acetylcholine receptors (ACh-R) by combined treatment with mecamylamine and atropine or treatment with strychnine (which blocks alpha9 ACh-R) for 5-10 days resulted in a complete inhibition of epidermal differentiation and proliferation. Blockage of nicotinic (n)ACh-R with mecamylamine led to a less pronounced delay in epidermal differentiation and proliferation than blockage of muscarinic (m)ACh-R with atropine, evidenced by reduced epithelial thickness and expression of terminal differentiation markers like CK2e, CK10, ZO1 or filaggrin. In OTCs treated with atropine, mecamylamine or strychnine we could demonstrate intracellular lipid accumulation already in the lower epidermal layers indicating metabolical stress and a severely disturbed epidermal barrier. In addition, we observed prominent acantholysis in the basal and lower suprabasal layers in both, mecamylamine and atropine treated cultures, accompanied by a decreased expression of desmoglein 3 and E-Cadherin. In contrast, stimulation of nACh-R and mACh-R with cholinergic drugs, resulted in a significantly thickened epithelium accompanied by an increase of intercellular lipid content in the corneal layer. CK2e, filaggrin and ZO1 showed a slightly extended expression in the upper epidermal layers, whereas proliferation was not significantly increased. In summary we showed that inhibition of epidermal ACh-R severely disrupts epidermal homeostasis. In particular, terminal differentiation, barrier formation, cell adhesion and proliferation are controlled by both nicotinic and muscarinic ACh-R.

V12

Mice deficient for the Lymphotoxin Beta Receptor (LTβR KO mice) are susceptible to L. major infection

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Lymphotoxinβ (LTβ) is essential for the development of the lymphoid system, as evidenced by a severely disrupted lymphoid architecture in mice deficient for either LTβ (LTβ KO) or the LTβ receptor (LTβR KO). Both mice show an increased susceptibility to infections which require a Th1 immune response for resolution. To clarify the mechanisms for increased susceptibility we compared the course of cutaneous infection with leishmania major (L.m.) in LTBR KO , LTβ KO, resistant wildtype (C57/Bl6) mice and susceptible Balb/c mice.
In agreement with data from Wilhelm et al. (1) LTβ KO mice showed an increased footpad swelling as well as an increased number of parasites at the site of infection though they developed a Th1 type immune response. The disease progression however was significantly delayed compared to BALB/c mice. In LTβ KO mice we also did - in contrast to Wilhelm et al (1) - not regularly observe parasite spread to liver or bone marrow as in Balb/c mice.
In LTβR KO mice however the disease progression was nearly identical to Balb/c mice and the parasites spread to liver and bone marrow. CD4 t-cells isolated from infected LTβR KO mice showed a TH2 type cytokine secretion pattern when stimulated in vitro with L.m. antigen pulsed dendritic cells. Transplantation of wildtype derived bone marrow in LTβR KO mice did not significantly alter the course of infection. This suggests that the absence of LTβR on leucocytes during infection is not the cause for Th2 differentiation and susceptibility.
In conclusion LTβR KO mice show a Th2 switch in L. major infection in contrast to LTβ KO mice. This switch is most likely due to an effect of LTβR on the development of the lymphoid system. Since LTβR KO mice completely lack any peripheral lymphnodes the Th cell differentiation can not take place in the draining lymphnodes as in wildtype mice. It is therefore tempting to speculate that the environment in which dendritic cell -
t-cell interaction takes place influences the outcome of Th cell differentiation. The presence of small cervical and mesenteric lymphnodelike structures in LTβ KO mice may explain the mitigated course of infection in LTβ KO compared to LTβR KO mice.
(1) Wilhelm et al. Eur J Immunol, 2002, 32(7)

V13

The HLA - haplotype determines TH1 and TH2 responses in melanoma patients

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A genetic MHC associated disposition to mount TH1 or TH2 immune responses is a well known phenomena in certain inbred mouse strains, but up to now has not been encountered in humans. Here we describe for the first time opposing TH1 and TH2 immune responses in vaccinated tumor patients that correlate with the expression of MHC class I HLA-A2 or -A1 alleles.
We have vaccinated stage IV melanoma patients with autologous peptide loaded DCs to a variety of melanoma antigens and, in order to strengthen the induction of CTL responses, concomitantly induced helper responses to keyhole limpit hemocyanin (KLH). Indeed, repeated presentation of KLH by peptide loaded DCs not only resulted in strong CD4+ T cell responses to KLH, but also enhanced peptide specific CD8+ T cell responses. In HLA-A2 patients, both, CD4+ T cell responses to KLH as well as peptide specific CD8+ responses could routinely be detected by ex-vivo ELISPOT analysis, whereas in HLA-A1 patients CD8+ T cell responses were hardly detectable ex-vivo. A more detailed analysis then led to the surprising finding, that CD4+ responses to KLH were dominated by IFN-y production in 4 out of 5 HLA-A2 patients, whereas 4 out of 4 HLA-A1 patients presented continously strong IL-4 reactions and a complete loss of IFN-y production after the 4th vaccination. One patient, expressing both HLA-A1 and HLA-A2 alleles, showed continously high IFN-y and IL-4 responses to KLH. The dominant TH1 response in HLA-A2 patients was also reflected by a significantly higher percentage of peptide specific CD8+ T cells of the effector phenotype (CD45RA+, CCR7-). The TH1 / 2 bias in our patients showed no correlation to expression of class II HLA-DR,-DQ or -DP alleles.
In aggregate these data suggest a hitherto not realized human genetic predisposition to TH1 and TH2 responses, that is associated with class I HLA-A alleles and may have a crucial importance for immunotherapeutic concepts in tumor patients.

V14

Tolerance to contact allergens is mediated via TNFα-receptor-2 signaling in CD8+ effector T cells of contact hypersensitivity.

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Epicutaneous application of subimmunogenic doses of haptens induce low zone tolerance (LZT) to contact allergens resulting in the generation of suppressor CD8+ T cells (Tc2) that inhibit the development of contact hypersensitivity (CHS), mediated by CD8+ Tc1 cells. Here, we used TNFα-/- and TNFα receptor-deficient mice to better characterize the role of TNFα during LZT. Tolerizing doses of the contact allergen TNCB (0.45 or 4.5 μg per site, 5 times per mouse) were applied epicutaneously. Tolerance induction in vivo was assessed by measuring the inhibition of ear swelling in CHS. Importantly, we found that LZT is TNFα-dependent as TNFα-/- and receptor-double-KO-mice failed to develop LZT. Interestingly, TNFR1-/- (p55-/-) mice showed normal LZT responses, whereas no induction of LZT was observed in TNFR2-/- (p75-/-) mice. These findings indicate that TNFα and its receptor p75 play an important role in LZT development. In addition, prevention of hapten-specific clonal expansion of lymph node cells and Tc2 cytokines IL-4 and IL-10, that are typically upregulated in LZT, did not occur in TNFα-/-, receptor-double-KO- and p75-/- mice after tolerization. Adoptive transfer of T cells from tolerized TNFα-/-, TNFR2-/- or WT mice that were injected vice versa into WT or TNFα-/-, TNFR2-/- animals demonstrated that TNFα and TNFR2 signaling is not required for the induction and function of CD8+ suppressor T cells of LZT. However, TNFα induced signaling via TNFR2 (p75) is essential for the effector phase of LZT where TNFα prevents from the generation of CHS effector T cells. Notably, reconstitution of TNFα-/- and TNFR2-/- mice with T cells of WT mice between tolerization and sensitization revealed that the expression of TNFR2 on CD8+ effector T cells of CHS is mandatory for the tolerance reaction to contact allergens. Thus, our data demonstrate a critical role of TNFα via TNFR2 (p75) signaling in the effector phase of LZT, in which it inhibits the generation of CHS-promoting Tc1 cells and consequently the development of CHS.

V15

Psoralen plus UVA (PUVA)-induced premature senescence in primary human dermal fibroblasts is mediated by Ataxia teleangiectasia mutated and Rad3-related kinase (ATR)

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Premature skin aging is a common side effect of therapeutical PUVA treatment which is widely used for the treatment of different skin disorders. Previously, we were able to show that a single irradiation of primary human dermal fibroblasts with 8-methoxypsoralen and UVA could induce a stress-induced premature senescent phenotype (SIPS) in human dermal fibroblasts with characteristic morphological changes, increased matrix metalloproteinase expression, and expression of senescence-associated ß-galactosidase. Very recently, it was shown that Ataxia teleangiectasia mutated kinase (ATM) and not ATR mediate telomere shortening-dependent senescence in human cells. In addition, it could be shown that ATR and not ATM mediates a DNA crosslink-induced S-phase checkpoint in human cells.
By using different psoralen derivatives, monochromatic and broad band UVA irradiation, we could now show that the induction of SIPS in human fibroblasts correlated with the potential of the derivatives or wavelengths to induce crosslinks at the DNA molecule. Two hours after DNA-crosslinking PUVA irradiation, we could detect an activation of ATR and not ATM in primary human fibroblasts, keratinocytes and lymphocytes. Silencing of ATR in fibroblasts by siRNA prior to PUVA treatment led to a reduction of cell viability to 10% at day 11 after irradiation, as compared to the cells treated with control siRNA and PUVA. Remaining cell viability and induction of SIPS in the surviving cells correlated with transfection efficacies and thus most likely reflects untransfected cells. Moreover, immunoflurescence studies revealed a dot-like nuclear pattern of ATR and ATR interacting protein (ATRIP)and a co-localisation with γ-H2AX foci in SIPS fibroblasts at 14 days after PUVA treatment, but not in the same cells that underwent replicative senescence after more than 60 population doublings. γ-H2AX foci in senescent cells have been shown to be telomere associated.
In summary, DNA-crosslink dependence of SIPS, ATR but not ATM activation after PUVA treatment and subsequent co-localisation with γ-H2AX foci provide evidence for an ATR-dependent, possibly telomere localized, checkpoint after PUVA treatment. Since many patients are treated with PUVA, the detailed understanding of molecular signalling pathways may help to further improve irradiation protocols and to minimize potential side effects of the treatment.

V16

Interaction of High-Invasive Melanoma Cells with Native Collagen I via β1 Integrin Regulates the Activity of Extracellular Cathepsin B

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Tumor cell invasion involves several cell-cell and cell-matrix interactions, which may regulate the expression and localization of the lysosomal cysteine proteinase cathepsin B (Cat B) and their natural inhibitors stefin A, B and cystatin C. Imbalances between Cat B and its inhibitors are proposed to promote malignant progression in various types of cancer. In the present study, we investigated the expression and localization of the protease and its inhibitors in high- (MV3), intermediate- (SKmel28) and low-invasive human melanoma cell lines (SKmel23, WM164) after interaction of these cells with the extracellular matrix component collagen type I. Cells were either cultured as monolayers on plastic culture dishes or in contact to monomeric or fibrillar collagen I. Neither the mRNA levels of Cat B nor the amount of intracellular protein changed after melanoma cell interaction to collagen I. Similar results were obtained regarding the inhibitors, since mRNA levels of stefin B nor cystatin C stayed unaffected. Stefin A was not detectable in any of the analyzed cell lines. In contrast, remarkable differences in protein expression and localization were observed. Compared to low-invasive cells, stefin B expression was dramatically decreased in high-invasive MV3 cells. Furthermore, only the high-invasive cells constitutively released ProCat B, when cultured on plastic. After contact of these cells to native collagen I fibrils, also both mature forms of the protease were detected extracellularly, whereas no extracellular Cat B was detected in supernatants of low-invasive cells. Predominantly, mature Cat B was redistributed extracellularly in a collagen I dependent process, since the release of Cat B was diminished in the presence of inhibitory antibodies against the β1 integrin chain, emphasizing the role of collagen I binding receptors in this context. In addition, also the expression of cystatin C was observed to be dependent on cell-collagen I interactions. These results strongly implicate a pivotal role of integrin mediated cell-matrix interactions for the regulation of Cat B localization and protease inhibitor expression.

V17

Overexpression of a putative ubiquitin c-hydrolase identified by subtractive hybridization in PUVA-senesced fibroblasts results in a senescence-like phenotype

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Premature ageing of the skin is a prominent side effect of psoralen plus UVA (PUVA) photochemotherapy, used for various skin disorders. Following PUVA-treatment fibroblasts undergo long-term growth arrest, increased expression of interstitial collagenase and senescence associated β-galactosidase reminiscent of replicative senescence. Since the molecular basis of the functional and morphological changes is unknown, we applied subtractive hybridization to isolate genes that might be involved in this senescent phenotype. Among several differentially expressed cDNAs, one cDNA of an unknown gene showed the highest change with a 6-fold induction post PUVA-treatment. Using RT-PCR and RACE (Rapid Amplification of cDNA Ends), we isolated a full length cDNA revealing a 53bp 5 - end untranslated region, a 3222bp open reading frame and a 2269bp 3 - end untranslated region. In addition, three transcripts with high homology were detected. Northern blot analysis revealed strong expression of this gene in postmitotic human tissues like brain and heart, with virtually no expression in proliferating cells. From the translated cDNA sequence, the first 400 amino acids show high homology to a putative murine ubiquitin c-terminal hydrolase releasing ubiquitin from ubiquitinated proteins. Stable overexpression of this novel gene in juvenile fibroblasts resulted in a premature senescent phenotype with enlarged cell size, cessation of proliferation, expression of senescence-associated β-galactosidase and of matrix metalloproteinase-1, and high levels of reactive oxygen species within several weeks in culture. As the de-ubiquitination enzymes are involved in biologically important processes including growth and differentiation, cell cycle progression, and signal transduction, the analysis of this unknown gene promise useful insights into the role of de-ubiquitination in stress-induced and replicative senescence and degenerative disorders of connective tissue.

V18

Differential regulation of T cell responses and innate immune responses by T cell-derived interleukin-10

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Interleukin-10 (IL-10) is an important regulator of immune responses secreted by a variety of cell types including macrophages, lymphocytes, epithelial and mast cells. IL-10 deficiency results in exaggerated T cell and innate responses. In order to elucidate the contribution of the various IL-10 secreting cell types to immune regulation we generated mice with selective inactivation of the IL-10 gene in various cell types. Immune responses of mice deficient for IL-10 specifically in T cells were analysed and compared to responses of animals with complete IL-10 deficiency. The T cell-specific IL-10 mutants spontaneously developed inflammatory bowel disease and exhibited enhanced contact hypersensitivity responses and lethal immunopathology upon infection with low virulent Toxoplasma gondii reproducing the deregulated T cell immunity of complete IL-10 deficiency. In contrast to complete IL-10 deficiency, however, cutaneous irritant responses were not enhanced in the T cell-specific IL-10 mutants. These animals also tolerated the systemic application of the same doses of LPS as wild type control mice which killed mice with complete IL-10 deficiency. Serum levels of TNF-a, IFN-g and IL-12 six hours after LPS injection were dramatically increased in mice with complete IL-10-deficiency but not in the T cell-specific mutants. Thus, the innate irritant and LPS responses must be subject to regulation by IL-10 derived from non-T cells.
Our results show that secretion of IL-10 from different cell types can serve different functions and have implications for the therapeutic application of the cytokine. Mutants with inactivation of the IL-10 gene in macrophages, keratinocytes, and B lymphocytes are presently being investigated for alterations of T cell or innate responses.

V19

Beta2 integrins define the destination of circulating naïve lymphocytes in secondary lymphoid and non-lymphoid organs

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The immune system relies on a sophisticated cellular organization based on a dynamic homeostasis and maintained by constant migration and redistribution of immune cells. Each cell type along with its lineage markers possesses a diverse set of additional surface molecules including chemokine and integrin receptors that distinctly encode migratory behavior. β2 integrin receptors are particularly prominent in mediating adhesive events that contribute to lymphocyte migration. Whereas naïve lymphocytes (CD62L+CCR7+) recirculate between secondary immune organs, antigen-experienced effector lymphocytes (CD62L-CCR7-) screen the peripheral non-immune organs.
The objective of the present study was to clarify the role of β2 integrins (CD11/CD18) in determining the migratory pattern of naïve lymphocytes. For this purpose, adoptive cell transfers were performed. Naïve cells were isolated from peripheral lymph nodes of wild-type (WT) and CD18null (CD18-/-) mice, stained separately with the cytoplasmic cell-tracker dyes CMFDA and CMRA, and injected intravenously at a 1:1 ratio into WT recipients. Redistribution of the labeled cells into lymphoid and non-lymphoid organs was analyzed. Lymphocyte suspensions or histological sections of peripheral/mesenteric lymph nodes, spleen, blood, bone marrow, liver and lungs were prepared at 3 or 18 h after the transfer. Additionally, staining for B and T cell markers (B220, CD4 and CD8) was carried out, and analyzed by flow cytometry or fluorescence microscopy. In contrast to WT lymphocytes, almost no CD18-/- cells could be detected in peripheral lymph nodes at either time points after transfer, regardless of their phenotype. In addition, a reduced accumulation of CD18-/- cells was observed in spleen, blood, mesenteric lymph nodes, Peyer#s patches and bone marrow. Strikingly, a considerable amount of CD18-/- cells was detected in lungs and liver 3 h after injection. Even after 18 h the prevalence of CD18-/- cells in lungs and liver was still higher, however a remarkable decrease of lineage markers was observed.
Collectively, our data show that β2 integrins are of central importance for defining the destination of naïve lymphocytes within immune and non-immune organs. Whether their affinity/avidity levels may be regulated by distinct chemokines in different organs will now be further investigated.

V20

Nej2: Novel telomere-associated proteins with inhibitory functions on DNA double-strand break repair

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Telomeric maitenance plays important roles in cellular processes as chronologic aging, photoaging and carcinogenesis. Structurally, telomeres are free DNA ends and thereby represent DNA double-strand breaks (DSB) that have to be protected from degradation or deletorious end-to-end ligations. Recent work has substantially elucidated dual, partially antagonistic functions of several proteins in telomeric maintenance that otherwise act in DSB repair and signalling. It could be shown that end-to-end ligations at telomers are mediated by DNA ligase IV and XRCC4, proteins that act in the DSB repair pathway of non-homologous end-joining (NHEJ) which is higly conseved between yeast and mammals. Searching for proteins that physically interact with Lif1p, the yeast orthologue of human XRCC4, we identified Nej2p (non-homologous end-joining associated 2), a previously unknown yeast protein in two-hybrid screens. Database searches have revealed a closely related human homologue, hNej2 and we established that this ubiquitously expressed protein indeed engages in a conserved protein-protein interaction with XRCC4. Three-hybrid studies with Lif1p, its interacting partner proteins DNA ligase IV (Lig4p), Nej1p and Nej2p, demonstrated a competitive role of Lig4p and Nej2p in forming complexes with Lif1p and a bridging function of Lif1p in a Nej1p/Lif1p/Nej2p complex. In vivo DSB repair assays in yeast cells overexpressing Nej2p implicate an inhibitory role for Nej2p in NHEJ with a preference for DSB with 3#-overhangs. Within cells, overhanging 3#-ends are found at the free ends of telomere structures. Indeed, localisation studies of yeast and human Nej2 showed that Nej2 localize in the nucleus with a "dot-like" pattern reminiscent of telomeric structures. Moreover, we could co-localize human Nej2 in human WI26 fibroblasts with telomere-associated proteins as telomere repeat factors Trf1 and Trf2, telomere associated DNA repair protein Mre11, and PML bodies. Taken together, Nej2 proteins represent novel factors that associate with telomere- and DNA repair- proteins and may act as inhibitors of NHEJ at these sites. Further studies are currently under way to study biological functions of Nej2 at telomere structures with focus on alterations observed in cancers or during cellular senescence, and in response to DNA damaging agents in human skin cells.

V21

Identification of susceptibility loci for the psoriasiform skin disease in a murine psoriasis model

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Psoriasis is a complex disease with both heritable and environmental factors contributing to disease onset and severity. A number of genetic loci, but no single gene could be identified by genome wide linkage scans so far. As has been shown for other complex diseases like rheumatoid arthritis and hypertension, animal models resembling human disease are valuable tools for identification of candidate genes. A CD18 hypomorphic polygenic PL/J mouse model with severe reduction of CD18 (β2 integrin) to 2 - 16 % of the wild-type results in the development of a skin disease resembling human psoriasis. However, no psoriasiform skin disease develops in C57BL/6J mice carrying the same hypomorphic CD18 mutation.
To identify chromosomal regions harbouring genes that apart from the hypomorphic CD18 mutation are responsible for the manifestation of the psoriasiform dermatitis a backcross between CD18 hypomorphic mice of the susceptible PL/J and the resistant C57BL/6J strain was performed. 343 mice of the (PL/J x C57BL/6J)F1 x PL/J generation were observed for their psoriasiform phenotype using an adapted PASI score revealing that in addition to the CD18 hypomorphic mutation one or two further genes are responsible for susceptibility to the psoriasiform dermatitis, while four or more genes are responsible for severity. Compared to males, females are more susceptible to the psoriasiform skin disease. (PL/J x C57BL/6J)F1 x PL/J mice were analysed in a genome-wide linkage analysis using microsatellite markers. Gene loci on chromosomes 10 (LOD score 7) and 6 (LOD score 3) were significantly linked to the psoriasiform skin disease suggesting that these chromosomal regions harbour genes that are responsible for the manifestation of the psoriasiform dermatitis.
For the first time, we have succeeded to identify gene loci playing a role in psoriasis in a murine model very much reflecting human disease. The identification of genes associated with psoriasis in this mouse model might contribute to further elucidate genes and mechanisms causally involved also in human psoriasis and may have general relevance for polygenic human inflammatory diseases.

V22

Development of an active disease model for epidermolysis bullosa acquisita in mice

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Epidermolysis bullosa acquisita (EBA) is a subepidermal blistering disease associated with tissue-bound and circulating autoantibodies to type VII collagen, a major constituent of the dermal-epidermal junction. Using an in vitro system, we previously demonstrated the blister-inducing potential of patients# autoantibodies to type VII collagen. More recently, we showed that rabbit antibodies generated against the NC1 domain of murine type VII collagen induce subepidermal blisters when passively transferred into mice. These models are useful to dissect the pathogenic mechanisms of blister formation induced by EBA antibodies. However, the induction and modulation of the autoimmune response to type VII collagen cannot be studied in these models. Therefore, in the present study, we attempted to develop a model of EBA reproducing the active disease by immunization of mice. Animals of 4 different strains, including BALB/c (H2d), FcgammaR2b-deficient (H2b), SJL-1 (H2s), and SKH-1 (outbred), were immunized with a recombinant form of the murine type VII collagen NC1 domain. All mice (n=20) developed circulating serum IgG autoantibodies to type VII collagen as determined by immunofluorescence and immunoblot studies. These antibodies exclusively bound to the lamina densa of mouse skin by immunoelectron microscopy. Levels of serum autoantibodies, evaluated at different time points by ELISA using recombinant antigen, were shown to be similar in all strains. Immunofluorescence analysis of murine skin revealed bright deposition of IgG at the dermal-epidermal junction. Importantly, subepidermal blisters developed in 60% of SJL-1 and 40% of BALB/c mice, but not in SKH-1 and FcgammaR2b-deficient mice. While strong complement C3 deposits were detected at the dermal-epidermal junction of perilesional skin in susceptible strains, complement fixation was weak or absent in non-responder strains. In conclusion, for the first time, we report an active disease model reproducing the clinical, histo- and immunopathological as well as ultrastructural findings of EBA. This animal model should be most helpful to study the autoimmune response to type VII collagen and to develop more specific immunomodulatory therapies for this and other autoimmune blistering diseases.

V23

Solar UVA radiation-induced signaling involves a ceramide-mediated autocrine loop leading to ceramide de novo synthesis

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We have previously shown that exposure of human epidermal keratinocytes to solar UVA radiation at physiological doses leads to increased gene expression such as ICAM-1, which is mediated through activation of transcription factor AP-2 (Proc Natl Acad Sci USA 93: 14586-14591, 1997). AP-2 activation and gene transcription follow a biphasic pattern with a 1st peak between 0.5 to 2 hours and a second, more sustained activation between 16 and 48 hours. More recently we have demonstrated that ceramide signaling constitutes the mechanistic basis of the biphasic nature of this UVA response. Accordingly, UVA radiation-induced signaling is initiated by non-enzymatic formation of second messenger ceramide 0.5 to 1 hour after irradiation (EMBO J. 19: 5793-5800, 2000). This early peak in ceramide formation is followed by a second peak between 16 and 48 hours, which is due to ceramide de novo synthesis through activation of serine palmitoyltransferase and of functional relevance because its inhibition abrogated the 2nd, but not the 1st peak in UVA radiation-induced gene expression.
Now we present evidence that this second peak results from a ceramide-mediated autocrine loop. Stimulation of unirradiated cells with cell permeable (C2, C6) ceramides induced (i) serine palmitoyltransferase expression and activity and (ii) subsequent ceramide formation. Even more important, in irradiated cells, inhibition of the 1st peak by strategies that did not interfere with ceramide de novo synthesis, consistantly inhibited the 2nd peak. Accordingly, pretreatment of keratinocytes with vitamin E (singlet oxygen quencher), cholesterol (inhibition of raft signaling) or the compatible solute ectoin did not affect C2-ceramide-induced ceramide de novo synthesis, but completely abrogated UVA-induced ceramide formation, AP-2 activation and gene expression at early and late time points. In conclusion we show for the first time that ceramide can act in an autocrine manner to stimulate the de novo synthesis of ceramide in mammalian cells. This newly identified autocrine loop forms the molecular basis of the UVA response in human keratinocytes.

V24

Induction of apoptosis in human melanoma cells by the proapoptotic Bcl-2-related protein Bik/NBK

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Proteins of the Bcl-2 superfamily located at the outer mitochondrial membrane are central regulators of apoptosis and include both antiapoptotic as well as proapoptotic factors. The proapoptotic BH3-only protein Bik/NBK was initially identified as a binding partner of E1B19K, Bcl-2 and Bcl-XL and inhibits the antiapoptotic activity of these proteins thereby supporting the death promoting ability of proapoptotic factors like BAX. In order to evaluate the suitability of NBK in an anti-melanoma approach, we investigated the effects after NBK overexpression in cultured melanoma cells and in a melanoma xenotransplant nude mouse model.
Melanoma cell lines investigated represented only weak basic expression of NBK at the mRNA and protein level. After transient transfection of NBK cDNA under control of a tetracycline-responsive promoter, apoptosis was significantly induced in tetracycline-regulatable melanoma cell lines derived from SK-Mel-13, Bro, Mel-2a and SK-Mel-19. In stably NBK-transfected SK-Mel-13 cell clones (SKM13-NBK) inducible overexpression of NBK also enhanced sensitivity to other proapoptotic stimuli as agonistic CD95 activation and chemotherapeutics as etoposide and doxorubicin. For investigating the effect of NBK overexpression in-vivo, SKM13-NBK cells were subcutaneously injected into nude Balb-C mice. For promoter induction mice received doxycycline with the drinking water whereas the control group was left untreated. Tumor growth was significantly delayed in doxycycline-treated animals 7 weeks after induction of NBK. By investigating the mechanism of NBK-induced apoptosis, SKM13-NBK cells were found to represent typical hallmarks of apoptosis such as DNA fragmentation and chromatin condensation, after induction. Interestingly, apoptosis seemed to be independent from other characteristic apoptosis markers as cytochrome c release and caspase activation.
These data indicate that the BH3-only protein NBK is a potent inductor of apoptosis in melanoma cells and may suggest strategies against malignant melanoma based on exogeneous NBK overexpression as a novel proapoptoic tool.

V25

INVESTIGATION OF SELECTIN-BLOCKING HEPARINS AS PROMISING ANTI-INFLAMMATORY AGENTS

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Selectin-mediated leukocyte rolling along the endothelial surface of blood vessels is of key importance for the cellular immune response, since rolling initiates the extravasation of leukocytes to sites of inflamed tissue. However, dysregulation might lead to an uncontrolled excessive infiltration into healthy tissue. Selectins are implicated in the development of pathological inflammations, such as rheumatoid arthritis, asthma, or chronic inflammatory dermatosis (e.g. psoriasis). Consequently, blocking of selectins has attracted much attention as a promising target for therapeutic interventions. Heparin was shown to efficiently block P- and L-selectin at therapeutic concentrations. Though, as a natural glycosaminoglycan heparin generally represents complex molecular mixtures and thus complicates clear structure-activity relationships. The aim of the present study was to get an insight into structural requirements of heparins for P-selectin inhibition. We compared four different fractionated and non fractionated heparins with a series of 17, newly synthesized, defined polysulfated glucans of semi-synthetic origin which all gradually differ in their degree of sulfation, molecular weight, sulfation pattern and chemical linkage of the monomers. The inhibitory capacity of compounds was monitored in a parallel plate flow chamber evaluating rolling of U937 cells on a P-selectin layer with respect to rolling fraction and rolling velocity. The inhibitory results were correlated with the structural characteristics and display that the degree of sulfation appears to be the most important parameter for inhibition, whereas molecular weight is of minor importance. Consequently, sulfated polysaccharides with optimal characteristics, such as members of the curdlan family strongly exceed the heparin blocking capacity. In order to confirm the P-selectin blocking capacity of a curdlan derivative (PS3) in vivo we investigated the P-selectin-dependent rolling of activated platelets along endothelial surface in murine skin vasculature using intravital microscopy. Our data shows that rolling of activated platelets is significantly reduced after treatment with PS3; rolling fractions: 46.9±20.9% and 23.4±12.9%, respectively. Hence, sulfated polysaccharides with optimal characteristics are potential promising candidates for the development of anti-inflammatory agents.

V26

The shedding of collagen XVII/BP 180 depends on plasma membrane microenvironment.

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Collagen XVII / BP180 represents a hemidesmosomal component and mediates the adhesion of the epidermal keratinocytes to the underlying basement membrane. It exists as full-length 180 kDa type II orientated transmembrane protein and as soluble 120 kDa ectodomain. The latter is proteolytically released from the cell surface by sheddases of the disintegrin and metalloproteinase (ADAM) family. Since collagen XVII as well as the ADAMs are membrane integrated proteins, the microenvironment of the plasma membrane can influence their shedding behavior. It is well known that several sheddases and their substrates share the affinity for lipid rafts and that their localization within the lipid rafts also influences the shedding process. Lipid rafts are microdomains of the plasma membrane enriched in cholesterol and sphingolipids. In this work, we had analyzed the shedding behavior of collagen XVII in HaCaT keratinocytes and collagen XVII transfected COS-7 cells in the presents of increased concentrations of the cholesterol-depleting agent methyl-ß-cyclodextrin (MßCD), which disintegrate lipid raft microdomains. As result, we observed increased collagen XVII shedding at a concomitantly decreased level of plasma membrane cholesterol. Filipin binding to the plasma membrane cholesterol, which prevents its incorporation in rafts, produces the similar inhibitory effect. This MßCD stimulated shedding was completely inhibited by sheddase specific inhibitors. Overexpression of the TNF-alpha converting enzyme (TACE) in collagen XVII / TACE co-transfected COS-7 cells revealed additional stimulated shedding under low-cholesterol conditions, indicating the involvement of TACE in this process. Our data show first evidences that the lipid plasma membrane organization plays an important role in regulation of collagen XVII shedding and therefore influence the regulation of keratinocyte migration and differentiation.

V27

A failing rescue mechanism in somatically mutated Patched Gene is associated with Gorlin-Syndrome

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Hedgehog pathway activity, regulating homeostasis of cell proliferation and differentiation in a variety of tissues, is triggered by stoichiometric binding of Hedgehog ligand protein (Hh) to the transmembrane transporter Patched (Ptc), an interaction that leads to relief of the associated regulatory protein Smoothend (Smo). Mutations of patched are implicated in the development of sporadic basal cell carcinomas (BCC) and Gorlin syndrome, an autosomal dominant disorder characterized by multiple skin and endodermally derived cancers as well as congenital abnormalities of the brain, bones and teeth. Due to presence of numerous putative splice variants of patched a firm genotype-phenotype correlation has not been established yet.
In a patient with Gorlin syndrome, we identified the novel missense mutation A1315S by heteroduplex analysis and subsequent direct sequencing of the genomic DNA extracted from PBMCs. In addition to this germline alteration, the stop codon G1019X and the missense mutation A1070S were detected in a BCC-sample of the same patient. Loss of heterozygosity within the tumor was determined by isolation of mRNA.
Despite the stop codon present in the BCC, real-time RT-PCR showed that the mutational inactivation of the gene led to an over-expression of patched mRNA owing to failure of a negative feedback mechanism in tumor cells compared to skin of a pool of normal individuals. Interestingly, the PTC stop mutation caused alternative splicing with skipping of mutation harboring exon 19 while no alterations of intron/exon boundaries within the splice-junctions were detected. The existence of the truncated protein was determined by a Western-Blot analysis. The data indicate a putative rescue-mechanism completely different to known splicing aberrations. A rescue attempt by skipping of the affected exon circumvents the nonsense mutation, however at cost of diminished functionality. The absence of unaffected protein end-product indicates the somatic inactivation of the second ptc gene allele resulting in the development of basal cell carcinoma.

V28

Mast cell-deficient and neurokinin-1 receptor knockout mice are protected from stress-induced hair growth inhibition

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Hair loss has often been reported to be precipitated by stress, conversely, insights on distinct mediators in the skin orchestrating the pathophysiological response to stress were long lacking. Recently, we have revealed the existence of a 'brain-hair follicle axis' by characterizing neuropeptide Substance P (SP) as a central element in the stress-induced threat to the hair follicle, resulting in premature onset of catagen accompanied by mast cell activation in the skin. Yet, our understanding of putative SP-mast cell interactions in the skin in response to stress was limited, since 1) the receptor through which SP may activate skin mast cells and 2) the extent of mast cell mediated aggravation of SP remained to be elucidated.
We now employed NK-1 receptor knockout mice (NK-1R-/-) and W/Wv mast cell-deficient mice, and observed that stress-triggered premature induction of catagen and hair follicle apoptosis does not arise in NK1 -/- and W/Wv mice. Further, the activation status of mast cells was reduced in stressed NK1 -/- mice, compared to the wild type control. Additionally, stress-induced up-regulation of SP positive nerve fibers was absent in both, NK-1R and W/Wv mice. These results indicate that the cross talk between SP and mast cell activation via NK-1R appears to be the sole most important pathway in the regulation of hair follicle cycling upon stress response.

V29

Microtubule-targeted drugs inhibit endothelial VEGF receptor-2 expression

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Cytoskeletal polymers control a wide range of fundamental cellular functions, including proliferation, migration, and gene expression. As complex changes in endothelial cell shape and motility are required to form new vascular networks, we hypothesized that disassembly of actin filaments or microtubules may impact endothelial VEGF receptor-2 (VEGFR2) expression as a critical determinant of angiogenesis. We therefore investigated the effect of actin filament- and microtubule-disrupting agents on VEGFR2 expression by human vein endothelial cells. While depolymerization of actin filaments (by cytochalasin D or latrunculin A) failed to induce discernable changes in VEGFR2 protein expression, microtubule (MT)-disassembly (by nocodazole, vinblastine, or colchizine) greatly inhibited endothelial VEGFR2 expression in a time- and concentration-dependent fashion. The suppressive effects of MT-targeted compounds on VEGFR2 expression were neither conveyed by an inhibition of endocytotic VEGFR2 recycling nor by an increase in lysosomal degradation. In addition, VEGFR2 inhibition was not mediated via augmented VEGFR2 shedding or shortened protein half-life, suggesting that transcriptional rather than posttranslational mechanisms account for the observed effects. In line with this conclusion, MT disruption significantly suppressed endothelial VEGFR2 mRNA accumulation. While analyses of mRNA half-lives did not reveal a decrease in VEGFR2 mRNA stability, treatment with MT-targeted drugs considerably suppressed transcriptional activity of 5'-deletional VEGFR2 promoter gene constructs. Hence, modulation of the rate of gene transcription represents the preferable molecular mechanism by which MT disruption inhibits VEGFR2 expression. Together, our data identify endothelial VEGFR2 expression as a novel target of MT-depolymerizing compounds. As antivascular effects of microtubule-targeted drugs are increasingly appreciated, enhanced insights in their modes of action are critical to define more optimal combinations with additional classes of therapeutic compounds.

V30

Proteinase-activated receptor-2 (PAR-2) prevents epidermal skin tumorigenesis

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Recent findings indicate a role of serine proteinases in tissue homeostasis as well as tumor development. Proteinase-activated receptors (PARs) belong to the family of G protein-coupled receptors. Receptor activation requires specific proteolytic cleavage of the extracellular N-terminus. So far, four PARs have been cloned: PAR-1, -3 and -4 are cleaved by thrombin whereas PAR-2 is activated by various trypsins, proteinase-3 and mast cell tryptase. The newly formed N-terminus, the "tethered ligand", is able to bind to the second extracellular loop of the receptor, leading to conformational changes and subsequent signal transduction. In vitro, PARs can also be stimulated by specific activating peptides that mimick the tethered ligand. In the skin, PAR-2 is produced by epidermal keratinocytes and several other cell types. Analysis of human squamous cell carcinoma tissue showed considerably reduced immunoreactivity for PAR-2 within the tumor area as compared to normal skin. This was consistent with northern blot analyses of PAR-2 mRNA in squamous cell carcinoma tissue derived from several patients: with increasing malignancy of the tumor, PAR-2 mRNA production was downregulated. In contrast, PAR-1 mRNA expression was upregulated during tumor dedifferentiation. In a mouse skin tumor model using chemically-induced carcinogenesis, PAR-2-deficient mice showed a significantly increased number of skin tumors (appr. 8-fold), compared to wild-type animals. To further investigate the molecular basis of these observations, possible signal transduction pathways activated upon PAR-2 stimulation in HaCaT keratinocytes were analyzed. Western blot analysis showed an involvement of ERK1/2 and profound EGF receptor transactivation. Inhibition studies revealed that EGFR transactivation was triggered by metalloproteinase activity. Finally, ELISA analysis showed that activation of PAR-2 led to secretion of the tumor suppressor TGF-β1, probably via EGFR activation. In summary, PAR-2 is involved in keratinocyte differentiation and may play a beneficial role for the treatment of epithelial skin tumors. Thus, serine proteinases are not only involved in tumor progression but also prevention of tumor progress, as shown in squamous cell carcinoma.

V31

In vivo suppression of regulatory T cells by adenovirus mediated transduction of dendritic cells.

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We have recently shown that dendritic cell (DC) specific anti-DEC-205 antibodies (aDEC) target to DCs in the lymph node after s.c. injection into mice. To utilize this unique targeting for selective in vivo gene transfer, we generated recombinant adenovirus encoding for a suppressive T-cell receptor mimic peptide (AdTCRpep) and coupled this adenovirus to aDEC antibodies. Upon injection into mice we could show that aDEC-AdTCRpep conjugates specifically transduced CD11c+ DCs in the lymph node. In contrast we could not observe expression of the AdTCRpep in B-cells, macrophages or T-cells. In vitro analysis of the transduced DCs revealed reduced T-cell stimulatory capacity, indicating that the inhibitory TCRpep is secreted in an active form. Since in vivo DCs in the steady state preferentially interact with CD25+ regulatory T cells (Treg), we reasoned that the majority of T-cells affected by AdTCRpep transduced DCs in vivo are Tregs. Therefore we isolated CD25+ T-cells from aDEC-AdTCRpep injected mice and analyzed their regulatory capacity in MLRs. Here we could show that Treg from injected mice failed to inhibit proliferation of allogeneic T cells, indicating suppression of their regulatory function. Moreover, in a contact hypersensitivity (CHS) model, injection of the aDEC-AdTCRpep conjugates augmented ear swelling reactions, presumably via the suppression of CD25+ Tregs in vivo as indicated by down regulation of the FoxP3 mRNA. In order to test the effect of aDEC-AdTCRpep induced Treg suppression on immunity in a B16 melanoma model, we treated mice with aDEC-AdTCRpep conjugates and challenged for tumor growth thereafter. In these experiments tumor growth was substantially slowed in conjugate treated mice as compared to untreated controls. Thus these data indicate that suppression of regulatory T cells in vivo counteracts their immunosuppressive function and turning off the "regulators" may be a future goal for the improvement of vaccination strategies.

V32

zurückgezogen

V33

CD28-mediated costimulation impacts on differentiation of DC-vaccination induced T cell responses

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Co-stimulatory signals are essential for effective T cell responses. In this regard the CD28/B7 receptor ligand system is one of the best characterized pathways. While CD28 seems to be important for the activation of naive T cells, the role of the latter in formation of anti-tumor immune responses is controversial. In the present study, we compared the efficacy of DC-induced melanoma specific, i.e. anti-Trp-2180-188/Kb T cell responses to control either subcutaneous or pulmonary metastases of B16 melanoma in CD28 k.o. mice. To this end, in either tumor model the tumors developed faster in CD28-deficient mice as compared to wild type (wt) animals. To scrutinize whether lack of CD28 signalling influences priming of CD8+ T cells, FCM analysis of spleenocytes of both genotypes were performed after two immunizations with Trp-2180-188-pulsed DC. Interestingly, wt and CD28-deficient mice demonstrated a substantial and comparable number of Trp-2180-188/Kb-specific CD8+ T cells in the spleen in response to DC vaccination. Immunhistological analysis demonstrated that these cells homed similarly in wt and CD28-deficient mice into the tumor. Analysis of the clonality of the respective TIL using the RT-PCR/DGGE-based clonotype mapping confirmed that the tumor reactive T cells indeed preferentially home to the tumor. Functional analysis of Trp-2-reactive cells, however, revealed that the number of IFN-gamma-producing cells was substantial lower in CD28 k.o. mice. Hence, CD28-mediated costimulatory signals have in our model system neither an impact on priming nor on homing of CD8+ T cells but seem essential for differentiation of functional tumor specific CD8+ T-effector cells.

V34

T-cell selection in tonsils of psoriasis patients

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Skin inflammation in psoriasis vulgaris is mediated by antigen-specific activation and expansion of T cells within the skin. In a subtype of patients psoriasis onset is triggered by tonsillitis caused by S. pyogenes. We compared the T-cell receptor repertoire (TCR) of psoriatic skin lesions, peripheral blood lymphocytes and tonsillar T cells of three tonsillectomized patients in whom recurrent sore throat had been associated with severe psoriatic flares. T cells from the tonsils were sorted according to the expression of the skin homing receptor, cutaneous lymphocyte-associated antigen (CLA).
RNA and cDNA were prepared from the different tissues using standard procedures. TCRBV-gene specific PCR was used to amplify the whole TCRBV-gene family. Fragment length of the PCR products was analyzed on an ABI prism gene sequencer using gene scan software. cDNA of TCRBV genes with evidence of clonal selection was cloned and sequenced.
TCR spectratypes of both, skin lesions and CLA-positive tonsillar T cells showed strong evidence of clonal T-cell selection in several BV gene families that was not evident within CLA-negative tonsillar T cells or PBL. In part, the TCRs selected in psoriatic skin and CLA-positive T cells were identical in length and contained identical TCR rearrangements. These results indicate that streptococcal tonsillitis may select skin homing T cells within the tonsils that become reactivated in the skin and subsequently induce the psoriatic skin inflammation.

V35

FOXP3 EXPRESSION IS DIRECTLY LINKED TO THE REGULATORY FUNCTION OF HUMAN DESMOGLEIN 3 (DSG3)-SPECIFIC T REGULATORY 1 (TR1) CELLS

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We have recently identified Dsg3-responsive Tr1 cells with remarkable inhibitory action on Dsg3-responsive autoaggressive T helper (Th) cells in vitro. Noteworthy, Dsg3-responsive Tr1 cell clones (n=9) from 5 donors were found to be positive for the transcription factor Foxp3 while 8 Dsg3-responsive Th cell clones derived from 4 patients with pemphigus vulgaris were not. Based on this disparate Foxp3 expression pattern of Tr1 and Th2 cells, we investigated the role of Foxp3 on the phenotype and function of the Dsg3-responsive Tr1 cells. These Dsg3-specific Tr1 cell clones were characterized by the secretion of IL-10, TGF-β and IL-5 upon Ag stimulation, Dsg3-induced inhibition of autoaggressive Th clones and their proliferative response to IL-2 but not to Dsg3 or mitogenic stimuli. Upon coculture with 0.2-0.4 μM Foxp3-specific liposomal 21mer antisense (AS) oligonucleotides (but not of sense oligonucleotides), the expression of Foxp3 by the Dsg3-responsive Tr1 cells was inhibited in a dose-dependent manner. A total of 4 Tr1 cell clones treated with 0.4 μM Foxp3-AS showed a proliferative response to Dsg3 and mitogenic stimuli which was increased by a factor of 2.5x and 4.6x respectively compared to the sense controls. Moreover, AS-treated Tr1 cells clones lost their ability to suppress the proliferative response of Dsg3-specific Th2 cell clones by a factor of 3.8. In addition, AS (0.4μM) treatment of the Tr1 cell clones completely inhibited expression of GITR and CTLA-4 and led to an induction of IL-2 production. In summary, Foxp3 AS treatment of Dsg3-responsive Tr1 cells induced phenotype and function similar to that of the Dsg3-responsive autoaggressive Th cells. These observations strongly suggest that Foxp3 expression is critical for regulatory cell function and that inhibition of Foxp3 expression may foster autoaggressive T cell responses.

V36

A role for Rac1 in epidermal wound healing in vivo

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Small GTP binding proteins of the Rho family have been shown to regulate important functions of epithelial cells like migration, spreading, cell- cell contact formation and proliferation. In order to investigate functions of the small GTPase Rac1 in epidermal keratinocytes in vivo we have generated transgenic mice that express mutant versions of Rac1 in the basal cell layer of the epidermis. Integration of mutant DNA and expression of the corresponding mutant proteins was verified using standard techniques of DNA and protein analysis. Transgenic mice were born at the expected Mendelian ratios and were viable and fertile. To our surprise, C57Bl6 mice expressing a dominant negative version of Rac1, N17Rac1, in the basal epidermal cell layer did not show an ouvert skin phenotype. Histological analysis of skin samples carried out up to date show strictly basal expression of the transgene but no further abnormalities. When transgenic mice expressing N17Rac1 were wounded, however, a highly disturbed pattern of epidermal regeneration became evident. Abnormalities include hypoplasia of the wound edges and limited migration of keratinocytes over the wound bed. We are currently analysing the mechanisms that account for these disturbances of wound epithelialization. Our results show for the first time that activity of Rac1 in basal epidermal keratinocytes is required for normal wound healing in vivo.

P001

Fluoroquinolone-associated anaphylaxis in spontaneous ADR reports

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Anaphylaxis has been reported associated with the intake of fluoroquinolone (FQ) antibiotics. According to pathophysiology such reactions may be immune-mediated (anaphylactic) or result from direct stimulation of effector cells (anaphylactoid). Anaphylactoid reactions, however, may occur after first intake since no sensitization phase is necessary. In Germany, numerous cases of suspected adverse drug reactions (ADRs) are reported spontaneously to the BfArM and are registered in a large ADR data base. The aim of the present study was to analyze all cases of FQ-associated anaphylaxis contained in the BfArM database in respect of previous exposition, time to onset and other determinants.

All FQ-associated cases of anaphylaxis, anaphylactic shock, anaphylactic/-toid reaction reported to the BfArM between 1993 and 2004 were identified and assessed with regard to correctness of diagnosis and causal relation. Further analyses were performed in defined subgroups.

172 cases reporting the aforementioned terms were identified. In 152 cases correctness of diagnosis and causal relation was considered at least as possible and further analyses were restricted to this subgroup. Administration of moxifloxacin was reported in 75/152 cases (49%), and this figure did not seem to be matched by a comparable high number of exposed patients. Levo-, cipro-, and ofloxacin accounted for 25 (16%), 21 (14%) and 17 (11%) of the 152 cases, respectively. Intake of other FQs was reported in 14/152 cases (9%). Occurence of the ADR after the first dose or within the first three days was reported in 63/152 cases (41%), but no information on pre-exposure with this or any other FQ was provided with these reports. In 20/152 cases (13%) the reaction occurred within the first three days and it was stated, that the respective FQ has never been taken before, although previous administration of a different FQ was not explicitly excluded. In 1/152 cases (0,7%) it was stated explicitly, that no FQ has ever been taken before.

Anaphylaxis appears to be an ADR of the class of FQs. Time to onset is compatible with an underlying anaphylactoid mechanism in a relevant number of cases. Differences in reported frequencies constitute a signal for the true difference and should be further investigated.

P002

Expression and function of CXCR1 and CXCR2 in human Langerhans cells

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IL-8 is one of the most ubiquitous chemotactic factors and a pleiotropic proinflammatory cytokine. It exerts its biological activities in dependence of the specific chemokine receptors CXCR1 and CXCR2. Langerhans cells (LCs) contribute to inflammatory and immune responses by migrating from skin to lymph nodes, but the expression and possible function of CXCR1/2 receptors has not been reported so far. We have therefore studied IL-8 receptors in isolated LCs and in LCs localized within the epidermis. Using the PCR technique, mRNA transcription of both CXCR1 and CXCR2 receptors was found in isolated LCs. Both proteins were detectable by FACS analysis in isolated LCs. It is remarkable that, in contrast to CXCR1, nearly all LCs stained positive for CXCR2. CXCR1, but not CXCR2, displayed a distinct intracellular receptor expression. In addition, both receptor proteins were detectable by double-immunofluorescence staining in LCs within skin sections. Cell cultivation in the presence of IL-8 had no influence of CXCR1 or CXCR2 expression. Activation of LCs by IL-8 induced cell migration whereas cell maturation remained unaffected. These data indicate that IL-8 is able to contribute to the first step of LC migration, from the epidermis into the dermis, that occurs in inflammatory and immune reactions of the skin.

P003

Inhibition of human IgE production by Allergen-DNA transfected dendritic cells

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Atopic/allergic diseases are characterized by Th2-dominated immune responses resulting in IgE production. DNA-based immunotherapies have been shown to shift the immune response towards Th1 in animal models. In further studies we showed that dendritic cells (DC) transfected with allergen-DNA are able to stimulate human autologous CD4+ T cells from atopic individuals to produce Th1 cytokines instead of Th2 cytokines and to activate IFN-γ producing CD8+ T cells. The aim of this study was to analyze whether DC transfected with allergen-DNA are also able to influence IgE production by B cells. For this purpose, human mature monocyte-derived DC from grasspollen allergic donors were transfected with an adenovirus encoding the allergen Phl p 1 and cocultured with autologous B cells, CD4+ T cells, and CD40Ltransfected L-cells.

Total IgE production of B cells receiving help from CD4+ T cells stimulated with allergen-transfected dendritic cells decreased dose-dependendly compared to stimulation with allergen protein-pulsed DC. Total IgG production was not affected in summary. Allergen-DNA transfected dendritic cells are able to direct the atopic/allergic immune response from Th2-dominance towards Th1 and Tc1 also resulting in decrease of IgE-Production.

P004

Sensitization through the skin leads to Th2 dominated antigen specific immunity and dermatitis

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Atopic Dermatitis (AD) is a chronic inflammatory skin disease and induced by skin homing Th cells. The majority of AD patients have high allergen-specific IgE-antibodies and Th2 cells. These antigen-specific Th2 cells can be isolated from early AD skin lesions, but it was not clear whether these Th2 cells directly mediate skin inflammation by antigen specific stimulation. Moreover, it has been a matter of debate whether skin barrier disruption in AD patients facilitates and amplifies Th2 sensitization. To this end, Balb/c mice were sensitized by either consecutive i.p. injections with Ovalbumin (OVA) or by repetitive applications of OVA-patches onto the skin. Analysis of OVA specific antibody production revealed high Th1 associated OVA-specific IgG2a and no Th2 associated OVA-specific IgE in i.p.-sensitized mice. In contrast, epicutaneously sensitized mice had very low OVA-specific IgG2a, but high IgE levels. Moreover, analysis of the Th phenotype demonstrated a highly polarized Th2 phenotype in the epicutaneously sensitized mice only. Procedure comparison experiments demonstrated that epicutaneous sensitization needed a gentle tape stripping inducing epidermal barrier disruption in order to achieve Th2 sensitization. No tape stripping or forced tape stripping failed to induce an OVA-specific Th2 phenotype and, interestingly, forced tape stripping leads to a Th1-phenotype and Th1-associated antibodies. An OVA-specific dermatitis could be elicited by repetitive epicutaneous application of OVA in the epicutaneously sensitized mice and was dominated by Th2 cytokines. The pathogenetic role of OVA-specific Th2 cells for skin inflammation was proven by adoptive transfer experiments into ear skin of naive mice. Only application of Th2 cells together with OVA (Th2/OVA) induced strong ear swelling responses, but not Th2 cells or OVA alone. In conclusion, our data demonstrate that Th2 cells directly mediate dermatitis after stimulation with their specific allergen and that a disrupted skin barrier facilitates the increase of Th2-sensitizations as seen in AD patients. Together, this indicates that proper treatment of AD is also of prophylactic value in respect to the patient#s "atopic career".

P005

Modified basophil degranulation test in drug allergy

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Many adverse drug reactions (ADR) appear to have an immunological aetiology but diagnostic tools remain limited, because little is known about the relevant immunogenic determinants of most drugs.Recognition of low-molecular-weight agents by antigen presenting cells depends on the formation of hapten-carrier conjugates or the binding of the haptens to cellular macromolecules.While beta-lactams are reactive substances and by virtue of attachment to carriers give rise to multiple different antigenic determinants, chemically inert substances like sulfonamides must undergo oxidative metabolism to form reactive groups.Enzymes involved in bioactivation are those of the P 450 system and the macrophage myeloperoxidase.We aimed to establish a novel in vitro test on basophils with metabolized forms of these two drugs.Degranulation of autologous basophils from peripheral blood was measured after incubation with concentrations from 100μg/ml to 1x10E-6μg/ml of unmodified drug and drug metabolized by myeloperoxidase using a commercially available kit for flow cytometric assessment of basophil degranulation.11 patients with immediate hypersensitivity to sulfonamides in history, 11 patients with β-lactam hypersensitivity as well as 11 and 10 healthy controls, respectively, were investigated.Mean age differed significantly between patients (44±14) and controls (25±3) and in both groups male/female ratio was 1/3.However, neither age nor sex was associated to test results.In the β-lactam group there was no difference between patients and controls when unmodified drugs were used but higher degranulation in the patients group when metabolized drugs were used.These differences were statistically significant at concentrations 0,01μg/ml, 1x10E-4μg/ml and 1x10E-6μg/ml.In the sulfonamide group patients showed a higher rate of degranulation at all but 2 concentrations of unmodified drug and at all concentrations of metabolized drug.However, statistical significance was only reached at 10μg/ml unmodified drug and at 0,1μg/ml metabolized drug.This test might be useful in immediate drug allergy.It is independent from the antigenic determinant and includes metabolized forms of the drug and can be used with the original pharmaceutical form.In the next step, greater groups of patients and controls need to be investigated to proof these data.

P006

Low doses of contact allergens induce systemic tolerance mediated by hapten-specific regulatory CD8+ T cells independent of the route of application

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Low zone tolerance (LZT), induced by epicutaneous application of low doses of contact allergens, requires the generation of CD8+ suppressor T cells that inhibit the development of contact hypersensitivity (CHS). As skin-associated antigen presenting cells are not involved in the induction of LZT and hapten-specific suppressor CD8+ T cells are virtually found all over the lymphatic system of epicutaneously tolerized mice, we suggested LZT to be a systemically induced state of acquired peripheral tolerance. To investigate the influence of the local environment of the application site on the induction of LZT, subimmunogenic doses of TNCB were administered orally, i.v. and epicutaneously and, subsequently, tolerance induction was assessed by measuring the inhibition of contact hypersensitivity reactions in vivo (ear swelling). Notably, dose-response curves demonstrated that low doses of TNCB (0.45, 4.5, 45 μg) applied orally or i.v. induced a tolerance reaction comparable to mice tolerized epicutaneously. In addition, in vitro analysis revealed the generation of IL-10 secreting regulatory CD4+ T cells which induce the development of suppressor CD8+ T cells. Those CD8+ T cells are characterized by a typical Tc2 cytokine pattern of LZT in mice receiving oral or i.v.-injected allergens as previously described for the epicutaneous model. Adoptive transfer experiments of T cells obtained from i.v., orally or epicutaneously tolerized mice into naïve recipients demonstrated the generation of allergen-specific CD8+ suppressor T cells of LZT, which inhibit the development of CHS-promoting Tc1 cells and consequently the manifestation of CHS. Thus, LZT represents a general mechanism of peripheral tolerance by which the organism deals with small amounts of allergens preventing the activation of the immune system.

P007

Local intranasal allergen specific immunotherapy efficiently inhibits allergic airway inflammation and induces regulatory T-cells in a murine asthma model.

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Background: Allergen specific immunotherapy is a cornerstone in the management of respiratory allergy. In patients, either subcutaneous or sublingual application is used with variable efficacy. Little is known on site-specific immunotherapy with regards to efficacy and side effects. Therefore, we examined the effects of local intranasal versus subcutaneous allergen specific immunotherapy in a murine asthma model.
Methods: BALB/c mice were sensitized intranasally with ovalbumine (OVA) and were treated subsequently with increasing doses of OVA (1ug-1mg) intranasally or alternatively subcutaneously for a 3 week period. After sensitisation and immunotherapy, mice were challenged with OVA intranasally. Bronchoalveolar lavages (BAL) were performed and checked for airway eosinophilia. In addition, cytokines in the BAL fluid as well as antigen specific serum IgE levels were analysed by ELISA. The number of IL-10 producing CD4+CD25+ T cells in the BAL fluid was measured using intracellular FACS staining.
Results: Intranasal immunotherapy reduced airway eosinophilia and decreased IL-5 in the BAL fluid back to background levels of non sensitised mice. In addition, OVA-specific serum IgE antibodies and IL-4 levels were significantly inhibited, whereas IFN-y and IL-10 levels in the BAL fluid were increased. This was associated with an increased number of IL-10 producing CD4+CD25+ T cells in the BAL fluid. In comparison to intranasal treatment subcutaneous immunotherapy only induced slight antiallergic effects.
Conclusion: Local intranasal immunotherapy reversed the antiallergic phenotype more efficiently than the well established subcutaneous route in a murine asthma model, and induced IL-10 producing CD4+CD25+ T cells. This underlines the potential of local application forms for allergen specific immunotherapy in men.

P008

Glycan-associated epitope sharing of insect venom and natural rubber latex allergens

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Background: IgE-double positivity for honeybee (HB) and yellow jacket (YJ) venom sometimes causes diagnostic difficulties concerning therapeutical strategies. Previous investigations revealed that IgE-positive human sera for both, HB and YJ venom, also bind to a broad range of plant allergens due to cross-reactive carbohydrate determinants (CCD).
Material and Methods: 135 patients with suspected stinging insect allergy and CAP FEIA- double positivity were investigated for specific IgE to additional CCD-containing allergens: whole extracts of timothy pollen, rape pollen, rubber latex, bromelain, and horse radish peroxidase (HRP). Sera positive for rubber latex IgE were further investigated with the recombinant latex components rHev b1, b2, b3, rHev b5, b6.01, b6.02, b8, b9, and b11. The corresponding patients were subsequently investigated for clinical relevance of the CAP-FEIA-results (questionnaire, skin prick test). Reciprocal inhibition assays with both venoms (100μg/ml) and HRP (500μg/ml) were performed.
Results: 36/135 patients were IgE-positive to both venoms only. 99/135 additionally reacted to CCD-carrying allergens. 76/99 CCD-reactive sera had specific IgE to rubber latex. 46 were used for CAP FEIA with recombinant latex components. 31/46 rubber latex-IgE-positive sera were negative for anti-recombinant-latex-IgE, 15/46 were IgE-positive for at least one recombinant component. 18/46 of the corresponding patients who were already available for a skin prick test with natural rubber latex and a questionnaire were negative in history and skin prick test. An additional reciprocal inhibition assay was performed for 20 sera with HB and YJ venom, respectively. 16/20 were negative for CCD-binding IgE, 6/16 showed a homologous but not heterologous reaction, indicating a true double sensitisation of those patients. Inhibition with HRP in 24 sera revealed 100% inhibition of anti-HRP-IgE binding, in 3/24 HRP completely inhibited IgE-binding to YJ, and in 11/24 sera to HB.
Conclusion: In cases of IgE-positivity to both insect venoms supplementary CAP FEIA with at least one CCD-containing as well as recombinant allergens, should be performed. Subsequent reciprocal inhibition is an essential diagnostic tool to specify cross-reacting CAP FEIA results.

P009

Identification of two homologous IgE-binding peptides derived from staphylococcal enterotoxin B (SEB) and from the human low-affinity IgE-receptor (CD23) which modulate T-helper 1 and T-helper 2 cytokine production

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The majority of patients with atopic dermatitis (AD) mount an IgE response to staphylococcal exotoxins (e.g. SEB) that can be grown from their skin. It was the initial aim of this study to identify the IgE-binding sequence of the SEB molecule. We found that IgE molecules from patients with AD bind to an amino acid sequence beginning at the N-terminal position 181 of the SEB molecule. Data bank analysis revealed that this sequence is highly homologous to an amino acid sequence from the lectin domain of the human low-affinity IgE receptor (CD23). The stimulation of peripheral blood mononuclear cells (PBMC) derived from healthy individuals as well as from patients with AD with the two peptides (SEBp and CD23p) showed that both molecules do not have superantigenic properties but modify the endogenously prestimulated cytokine production of T-helper 1 (Th1) and Th2 lymphocytes. CD23p induced proliferation and IL-10 synthesis of Raji cells. On the other hand both peptides inhibited proliferation of Jurkat cells but only SEBp stimulated IFNg production. Additionally, in ovalbumin (OvA) sensitized BALB/c mice, SEBp inhibited allergen specific IgE and IgG2a synthesis, whereas CD23p stimulated the production of IgE and IgG1. Airway responsiveness after OvA exposure was increased after intravenous injection of CD23p. SEBp and CD23p markedly reduce the number of lymphocytes in the broncheo-alveolar lavage (BAL). SEBp suppressed the number of macrophages and eosinophils in BAL, whereas CD23p inhibited completely the infiltration of neutrophils. The results indicate that these nonapeptides could be a new group of immunomodifiers for the therapy of diseases which are characterized by an imbalance of the Th1/Th2 cytokine network, e.g. atopic dermatitis.

P010

Induced Eotaxin mRNA-Expression in Atopic Dermatitis-Derived Cultured Dermal Fibroblasts Indicates Enhanced Fibroblast Responsiveness to IL-4

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The presence of eosinophils and/or eosinophil derived products in the dermis is characteristic for involved skin areas of patients with atopic dermatitis (AD) and is believed to be responsible for the observed tissue injury. Eotaxin is a potent chemoattractant and activator of human eosinophils and IL-4 is a potent inducer of eotaxin-expression in dermal fibroblasts. Since increased fibroblast eotaxin expression may explain eosinophilic infiltration of involved skin areas in AD, we asked, whether dermal fibroblasts from atopic lesions differ in their ability to express eotaxin from normal fibroblasts. To address this question we cultured dermal fibroblasts derived from biopsies obtained from normal (4), chronic-lesional (5) and acute-lesional (4) atopic skin and compared IL-4-induced eotaxin mRNA expression using gel-based and real-time RT-PCR.

We found significant variability in IL-4 induced fibroblast eotaxin mRNA expression when comparing fibroblasts derived from different biopsies of the same group. The EC50 of IL-4 induced eotaxin mRNA expression was found to be lowest in fibroblasts originating from acute inflamed atopic lesions, when compared to fibroblasts obtained from chronic atopic lesions or normal skin. The variability in eotaxin expression in fibroblasts cultured from different AD patients may indicate heterogeneity of factors determining atopic phenotype in AD. The lower EC50 of IL-4-induced eotaxin-expression in fibroblasts originating from acute-inflamed atopic skin-lesions may indicate a special role for IL-4-induced dermal fibroblast eotaxin-expression in these lesions.

P011

Proteomic analysis of diferentially expressed proteins in urban and rural birch pollen grains

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Epidemiological studies reveal a correlation between the increase of allergies and exposure to environmental pollutants. Former studies focused on the impact of pollutants on the human immune system favouring an "allergic" immune response. Up to now no studies investigated the effect of environmental stress on the allergen carrier itself such as pollen grains. The aim of this study was to characterize the impact of anthropogenic factors on the protein expression in pollen grains. Since the individual is not only exposed to specific proteins such as allergens but rather to the whole pollen grain or its granules we focused not only on allergens but rather examined the entire pollen proteome utilizing the novel 2D eletrophoresis technology difference gel electrophoresis (DIGE).
Birch pollen grains were collected from trees at various sites in Munich city (urban, n=4) and in the surrounding area (rural, n=4). The urban and rural sites had substantially different levels of environmental exposure, established by measurement of the nitrogen dioxide concentration (an indicator for traffic emission). To estimate the pollution exposure of each tree, a traffic score was developed, based on the distance of the tree from the road. For the comparative proteome study of differently exposed birch pollen grains we used the difference gel electrophoresis (DIGE). Pollen grains were collected directly from the floral anther und extracted with 7M urea, 2M thiourea and 4% CHAPS followed by 2D electrophoresis.
With DIGE we found 34 differences in protein expression levels between the two groups (rural versus urban) at a $p \leq 0.05$ level analysed with DeCyder software. Eight proteins of interest were de novo sequenced from which one was identified to play a major role in the regulation of lipid metabolism such as oxygenation of the linolenic and linoic acid.
In summary we clearly show an impact of air pollution on the protein expression in pollen grains not effecting the expression of known allergens.

P012

The IgE-bearing B-cell receptor repertoire of atopic individuals shows unbiased VH-usage but patient-specific clonal expansions regardless of serum IgE levels

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Previous studies have postulated a bias in the usage of IgE-associated VH-chains in atopic individuals favouring a preferential usage of the VH6 segment. We have analyzed CD19-sorted B-lymphocytes from 15 adult atopic eczema patients displaying very high serum IgE levels (>1000 IU/ml). These purified B-cells were screened in a quantitative Real-Time RT-PCR technique for their transcription of all eight VH-families together with CH-primers of the ε-, γ1-4-, and μ-type, representative for the repertoire of IgE-, IgG-, and IgM-families. The VH-CHe amplifications were analyzed for their complementary determining region 3 (CDR3) spectra by a modified Immunoscope technique. Clonal expansions were further sequenced and compared to corresponding expansions of the IgM- and IgG-transcripts in order to screen for somatic mutations of the expanded clones and/or commonly selected clones. Serum IgE levels and antigen-specific IgE were determined using the IMMULITE® system.
Our approach revealed dominant usage of VH3b-, VH4-, and VH1-chains in IgE-producing B-cells as known for other Ig-transcripts regardless of the individual sensitization pattern. These chains were also preferentially found in IgG and IgM transcripts. Each patient however, harbored specific clonal expansions which were identified by the CDR3-spectratyping and which were not shared between patients even in cases of highly similar specific IgE production. Extensive sequence analysis revealed unique CDR3-sequences showing somatic mutations. However, sporadic clonal sequences were shared between IgE- and IgG transcripts suggesting a common origin.
Atopic individuals with increased IgE-levels show a highly individual pattern of IgE-bearing B-cell expansions with no preferential VH-usage but particular CDR3-composition.

P013

M-DC8+ dendritic cells as principal producers of interleukin 12 and their control by erythrocytes

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Interleukin 12 (IL-12) is a crucial cytokine for initiating innate immune responses and for programming Th1-dominated adaptive immune responses. Here, we identify the population of 6-sulfoLacNAc expressing proinflammatory human blood dendritic cells (DC) characterized by the mAb M-DC8 as the early and major source of IL-12 among human blood leukocytes and demonstrate their functional inhibition by erythrocytes. Starting with PBMC we noticed, that in order to produce significant levels of IL12p70, the cells required an initial six hour culture period prior to stimulation with the TLR4-ligand lipopolysaccharide (LPS). Single cell analysis among PBMC and stimulation of purified cell populations revealed that M-DC8+ DC but not monocytes or CD1c+ DC (DC1) acquired within six hours the capacity to produce the p70 form of IL-12 after stimulation with LPS or CD40-ligand alone. Priming with IFN-g also enabled IL-12 production by DC1 and monocytes, however, these levels were ten times lower compared to that of M-DC8+ DC. Furthermore, IL-12 production of PBMC was reduced to 1/9 when PBMC were depleted of M-DC8+ DC prior to stimulation.
The rapid aquisition of an IL-12-producing capacity by M-DC8+ DC was paralleled by the neoexpression of CD83 and CD80 and the up regulation of CD86 and CD40. These phenotypic changes were absent in whole blood cultures and also when coculturing M-DC8+ DC with erythrocytes. Finally, we demonstrate that culturing purified M-DC8+ DC with erythrocytes completely prevented their functional maturation as observed by their failure to produce IL-12. Taken together, controlling maturation and early IL-12 production appears to be a new biological role of erythrocytes that prevents M-DC8+ DC from untimely and possibly fatal responsiveness while circulating in blood. Furthermore, once M-DC8+ DC leave the vasculature and immigrate into the dermis, as observed upon local inflammation, they may serve as a particularly rapid and rich source of IL-12.

P014

Strain-dependent differences in inflammatory skin infiltrates during sensitization with contact allergens

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Contact hypersensitivity (CHS) is known to be a T cell-mediated immune response of the skin. Strain-dependent differences in the degree of inflammation of BALB/c versus C57BL/6 mice have been known for many years: BALB/c mice can be sensitized more easily than C57BL/6 mice, whereas stronger tolerance to contact allergens is observed in C57BL/6 mice. In contrast to the challenge phase, not much is known about the exact events during sensitization yet. One goal of this study was therefore to investigate the inflammatory infiltrate in mouse ears sensitized with 450 μg 2,4,6-trinitrochlorobenzene (TNCB) or vehicle. Ear swelling was measured over the course of 72 hrs, inflammatory cells were isolated after enzymatic digestion and mechanical disruption of ear skin and characterized by FACS every 24 hrs. As expected, ear thickness in BALB/c mice was increased compared to C57BL/6 ears. In parallel, more inflammatory cells were harvested from TNCB-treated BALB/c compared to C57BL/6 ears (23.5±1.6 vs. 10.9±1.5±10E6/ear, 24 hrs, n=6, $p \leq 0.002$). During sensitization the number of neutrophils increased more rapidly in BALB/c than in C57BL/6 mice. The total number of Gr-1+ cells in BALB/c was ≥2-fold higher at day 1 post sensitization in BALB/c compared to C57BL/6 ear skin ($p \leq 0.002$). Interestingly, the number of MHC II+ cells was also higher in BALB/c than in C57BL/6 ears during sensitization (24 hrs: 2.0±0.2 vs. 0.8±0.2x10E4/ear, n=6, $p \leq 0.002$, and 48 hrs: 2.5±0.3 vs. 1.2±0.4x10E4/ear, n=6, $p \leq 0.05$). During sensitization, influx of CD8+ T-cells was observed as early as 24 hrs and, interestingly, no strain-dependent difference was detected. Immigration of CD4+ T-cells was delayed and first observed in BALB/c ears starting at 24 hrs after TNCB application, whereas in C57BL/6 ear skin CD4+ T-cells were first detectable at 48 hrs and later. We next examined whether strain-dependent differences in the number of activated/regulatory CD25+ T-cells were detectable. During sensitization, the majority of CD4+ or CD8+ T-cells were CD25+ (~65%) and no strain differences were found. In summary, both the number of neutrophils and MHC II+ cells were increased in sensitized BALB/c compared to C57BL/6 ears. Strain-dependent differences in the inflammatory infiltrate of sensitized skin might therefore contribute to the degree and quality of sensitization against haptens.

P015

Chemokine Responses Distinguish Chemical-Induced Allergic From Irritant Skin Inflammation

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Chemical-induced adverse effects such as contact allergy as well as irritancy are of major importance in clinical dermatology and during the development of new pharmaceuticals or industrial chemicals. Since clinical and histological features of allergic and irritant contact dermatitis are similar, the differentiation between both types of dermatitis in the preclinical and clinical evaluation of chemicals remains difficult. However, the underlying immunological mechanisms are thought to be fundamentally different. Here we systematically studied the involvement of chemokine superfamily members (n=45) in the pathogenesis of both chemical-induced allergic and irritant contact dermatitis in mice and humans. Chemokines are small cytokine-like molecules binding G-protein coupled receptors and have been shown to critically regulate leukocyte trafficking. Comprehensive analyses of the chemokine and chemokine receptor expression during chemical-induced allergic and irritant skin responses indicated that hapten-induced murine contact hypersensitivity represents a valid model for human allergic contact dermatitis at the molecular level when compared with nickel sulfate patch test or genuine allergic contact dermatitis lesions. Notably, the expression of CXCR3 ligands such as CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (ITAC) distinguished between chemical-induced allergic and irritant skin responses in both murine and human models of contact dermatitis. Furthermore, we could show that these inflammatory chemokines synergize with the homeostatic chemokine CXCL12 (SDF-1) in recruiting skin homing CLA+ memory T cells. Taken together, findings of the presents study provide important insights into the molecular basis of chemical-induced allergic and irritant contact dermatitis, identify novel markers for their differentiation and demonstrate the cooperation of inflammatory and homeostatic chemokines in the recruitment of pathogenic leukocyte subsets.

P016

CCL18, an atopic dermatitis associated and dendritic cell-derived chemokine, is regulated by staphylococcal products and allergen exposure

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Atopic dermatitis represents a chronic inflammatory skin disease with a steadily increasing prevalence. Exposure to allergens or bacterial superantigens triggers Tand dendritic-cell recruitment and induces atopic skin inflammation. Here, we report that among all known chemokines CCL18/DC-CK1/PARC represents the most highly expressed ligand in atopic dermatitis. Moreover, CCL18 expression is associated with an atopic dermatitis phenotype when compared to other chronic inflammatory skin diseases. Dendritic cells either dispersed within the dermis or clustering at sites showing perivascular infiltrates are abundant sources of CCL18. In vitro, microbial products including lipopolysaccharide, peptidoglycan, and mannan as well as the T cell-derived activation signal CD40L induced CCL18 in monocytes. In contrast to monocytes, monocyte-derived interstitial-type and Langerhans-type dendritic cells showed a constitutive and abundant expression of CCL18. In comparison to Langerhans-cells, interstitial-type dendritic cells produced higher constitutive levels of CCL18. In vivo, topical exposure to relevant allergen or the superantigen staphylococcal enterotoxin B resulted in a significant induction of CCL18 in atopic dermatitis patients. Furthermore, in non-atopic NiSO4-sensitized individuals, only relevant allergen but not irritant (SLS) exposure resulted in the induction of CCL18. Taken together, findings of the present study demonstrate that CCL18 is specifically associated with atopic and allergic dermatitis, and it is expressed at the interface between the environment and the host by cells constantly screening foreign antigens. Its regulation by allergen exposure and microbial products suggests an important role for CCL18 in the initiation and amplification of atopic skin inflammation.

P017

Stress and experimental allergic dermatitis elicit differential c-Fos immunoreactivity patterns in the hypothalamus of the mouse

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Stress and inflammation are thought to share a number of common pathways and alter each others impact on the individual. In atopic patients the excitability of the hypothalamus-pituitary-adrenal (HPA) stress response system is decreased and may be involved in the pathogenesis and worsening of atopic dermatitis. However, whether stress and atopic dermatitis elicit additive or differential activation of central stress-response elements such as the hypothalamus has not been determined to date. Here we employed a mouse-model for stress (24h noise exposure) and experimental allergic dermatitis (AD) in mice to determine activation of the hypothalamus by quantification of c-Fos protein in the paraventricular and dorsomedial hypothalamic nuclei 48hrs after termination of stress-exposure, since at this time point the full picture of atopic dermatitis-like AD has developed in mice. We found, that stress significantly increased the number of c-Fos immunoreactive cell nuclei in unsensitized as well as AD mice in all hypothalamic nuclei investigated. Interestingly, in unsensitized mice, the stress-response was stronger in the left hemisphere - contra lateral to the side of the cutaneous inflammation - while in the AD mice the stress-induced increase did not differ between left and right hemisphere. Thus, in the left hemisphere stress+AD mice showed significantly less c-Fos immunoreactive cell nuclei compared to stressed mice. By contrast, AD mice that were not exposed to stress did not differ from control mice with the exception of the left dorsomedial hypothalamus, where the number of c-Fos immunoreactive nuclei was increased upon stress-exposure. We therefore conclude, that the stress-response can be provoked in AD mice and that AD by itself elicits a stress-response very distinct from noise-stress. However, an increase in dorsomedial activation by contralateral cutaneous inflammation may reduce paraventricular activation under stress and thereby reduce excitability of the HPA axis in AD mice under stress.

P018

Pollen provide a signal for human dendritic cells to modulate their chemokine and chemokinereceptor expression

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The immune response of atopic individuals against allergens is characterized by increased levels of Th2 cytokines and chemokines. However, the way in which the cytokine/chemokine profile is matched to the type of invading allergen, and why these profiles sometimes derail and lead to disease, is not well understood. We recently demonstrated that pollen grains not only function as allergen carriers but are also a rich source of pollen associated lipid mediators (PALMs) modulating dendritic cell function that results in an enhanced capacity to initiate T-helper (Th) 2 responses in vitro. Here, we examined the effects of APE on chemokine-receptor expression and chemokine production by DCs. APE (eluted over polymyxin columns to deplete LPS) strongly induced expression of CXC chemokine receptor 4 on human monocyte derived DCs while CD83 expression remained low compared to LPS. In contrast, APE reduced CCR1 and CCR5 expression on immature DCs. Furthermore, APE significantly reduced LPS-induced production of interferon-inducible protein 10 (CXCL10) and regulated upon activation, normal T-cell expressed and secreted chemokine (CCL5); increased secretion of macrophage-derived chemokine (CCL22); and did not significantly change production of thymus and activation-regulated chemokine (CCL17). Consistent with these findings, supernatants from APE together with LPS-treated mature DCs attracted Th1 cells less efficiently compared to LPS-treated DCs, while migration of Th2 was enhanced by APE-treated DCs. Our data suggest that pollen derived factors provide a signal for enhanced lymph node localization of DCs and that it may, at the same time, enhance the capacity of DCs to amplify type 2 immune responses by not only inducing a Th2 cell responses but also attracting preferentially type 2 cells.

P019

A new secretory organelle in human lymphocytes is involved in the pathophysiology of the atopy syndrome: Identification, isolation and characterization of RANTES-granules.

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The beta-chemokine RANTES (CCL5) plays an important role in the pathophysiology of the atopy syndrome. We now demonstrate that, in addition to de-novo synthesis and subsequent secretion, RANTES is stored in an unique pan-lymphocytic granule system. The discovery, characterization, isolation and release kinetics of this RANTES-granule system is described by flow cytometry, laser scan microscopy, ELISA, sucrose density gradients, western blotting and activation of cells using various stimuli and various inhibitors. RANTES-granules did not colocalize with known markers of established secretory organelles. They were of different density than secretory lysosomes containing granzyme-B. RANTES-granules were mobilized to the cell surface within one hour after an appropriate secretory stimulus like a combination of phorbol ester and calcium ionophore, and/or anti-CD3 crosslinking in the case of T cells. This stimulation-triggered release was independent of protein neosynthesis and was significantly faster as known secretory organelles of lymphocytes like lytic granules of cytotoxic T cells or natural killer cells. Investigating the peripheral blood from atopic patients, their RANTES-granule system was found to be significantly altered as compared to healthy individuals, namely a RANTES-hyperreleasability was detected. Taken together, a new secretory cell organelle in lymphocytes is described, filled with RANTES as the so far sole known cargo, which might expand our understanding of lymphocyte biology in general. In addition, the discovery of a RANTES-hyperreleasability in atopic patients supports the idea of a cell type independent defect of secretory granules as a major factor in the pathogenesis of atopy syndrome.

P020

Expression and function of TNF receptors on human basophils

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TRAIL and CD95 ligand (FasL) mediate apoptosis in different cell types by interaction with their specific receptors on the cell surface. We therefore hypothesized that TRAIL and CD95 ligand might also regulate survival of human basophils. Using FACS analysis, we examined the expression of TRAIL receptor-1 (TRAIL-R1), TRAIL-R2, and CD95 on the basophilic precursor cell line KU-812 as well as on basophils. Viability and apoptosis of both cell types was assessed by FACS analysis of PI uptake and binding of annexin-V, respectively. KU-812 cells expressed high levels of TRAIL-R1, TRAIL-R2, and CD95, whereas primary basophils expressed only low levels of TRAIL-R1 and TRAIL-R2, but showed also marked expression of CD95. In KU-812 cells, incubation with TRAIL significantly reduced the viability in a dose- and time-dependent manner. In addition, KU-812 cells were found to undergo apoptosis in response to TRAIL. The viability of basophils was not affected by TRAIL. Inhibition of protein synthesis by actinomycin D enhanced TRAIL-mediated apoptosis of basophils. In contrast, anti-CD95 was found to induce apoptosis of basophils, but failed to affect survival of KU-812 cells, even in the presence of actinomycin D. Our findings indicate that TRAIL and anti-CD95 participate in the regulation of basophil survival and may help to develop new strategies for the treatment of allergic disorders.

P021

Ex vivo isolation and characterization of CD4+CD25+ regulatory T-cells during specific immunotherapy in IgE-mediated allergy against inhalant allergens.

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Specific immunotherapy (SIT) is the only causative treatment of IgE-mediated allergic diseases. Although this therapy has been practiced for almost 100 years, the underlying mechanism is not fully understood.
The aim of this study was to investigate the role of CD4+CD25+ regulatory T-cells in the induction of allergen specific tolerance during SIT. The allergy vaccine applied contained a tyrosine-adsorbed chemically modified allergen extract from birch and the adjuvant monophosphoryl lipid-A (MPL®), a detoxified endotoxine of Salmonella minnesota. The specific sensitization profile of each birch pollen allergic was determined by immunoblot-analysis on SDS-separated natural birch pollen extracts. From blood samples (taken at defined intervals during SIT) PBMC were isolated and separated by MACS®. The percentage of the CD4+CD25+ T-cells in the PBMC were analysed by FACS. Using mature dendritic cells (mDC) presenting rBet v 1 the specific proliferative potential of bulk CD4+ T-cells, CD4+CD25+ and CD4+CD25- T-cells was analysed. In contrast, the unspecific proliferative potential was analysed by using plate-bound anti-CD3- and soluble anti-CD28-antibodies. The inhibitory potential of CD4+CD25+ T-cells and the cytokine secretion patterns of the three cell populations was investigated. Immunoglobulin titers (IgE, IgG, IgG4) specific for either rBet v 1, rBet v 2, rBet v 4 or the entire birch pollen extract were analysed during the course of SIT.
Participants sensitized exclusively against rBet v 1 showed an increasing percentage of the CD4+CD25+ T-cells in the PBMC during the course of SIT. In contrast, participants sensitized to several birch pollen allergens did not. All three cell populations (bulk CD4+, CD4+CD25-, CD4+CD25+) proliferated stronger after antigen specific stimulation by mDC presenting rBet v 1 in comparison to mock stimulation by mDC without antigen. Independent of the way of stimulation (with or without rBet v 1) the CD4+CD25- T-cells showed a stronger proliferation rate than the bulk CD4+ T-cells. During SIT serum levels of rBet v 1 specific IgG4 increased, whereas the level of rBet v 1 specific IgE remained unchanged.
In conclusion, CD4+CD25+ regulatory T-cells may play an important role in the development and treatment of allergic diseases.

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P022

Novel glove-derived proteins induce allergen-specific IgE in a mouse model

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Due to an increase of latex sensitizations in exposed risk groups, currently most latex products are produced at low content of latex proteins. However, recently foreign protein has been found in latex products. The substitution of genuine latex proteins by foreign proteins (e.g. casein or soy) may lead to a shift and a new increase in sensitization.
Aim of this study was to investigate the allergenicity and immunogenicity of different allergen sources (latex, soy and casein) in a mouse model.
Protein extracts were obtained by standard aqueous extraction and analysed qualitatively (SDS-PAGE, IgE- and IgG1-immunoblot) and quantitatively (Lowry protein assay, antigen specific IgE and IgG1-ELISA).Sixty BALB/c mice were repetitively s.c.immunized in groups of ten animals with equal amounts of either one of three natural rubber glove extracts, soy extract or casein each adsorbed to Al(OH)3 as adjuvant. As control, one group received solely the adjuvant. Immunization and bleeding of the animals were done in parallel initially in weekly, then monthly intervals over 196 days.
Allergen-specific IgE and IgG1-antibody responses were analysed by ELISA and statistically evaluated by analysis of variance. Antibody binding was investigated by immunoblotting and was further approached by microarray technique.
All allergen sources (latex, soy and casein) induced specific IgE and IgG1-responses. Allergen-specific human and murine antibodies are directed against allergens of similar molecular weight. Highest IgE-induction was rapidly observed after onset of immunization with two of the natural rubber allergen extracts. A protracted IgE-induction was observed with the third latex extract, soja and casein. By microarray technology clear IgE responses against kappa casein could be detected in mice immunized with a certain extract from a natural rubber latex glove presenting a hidden casein source.
In this study, we were able to establish a new immunological method based on micro-array technology to determine allergen-specific murine Ig-E antibodies. Foreign proteins were detected in latex products. Based on the results of a pronounced protracted IgE-induction by such substitutional proteins in the presented mouse model, a shift of elicitating allergens in "glove-related allergy" can be anticipated.

P023

Expression and purification in different expression systems of a high molecular birch pollen allergen showing pectinesterase activity

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Pectinesterases are a family of enzymes involved in degradation of pectin, a component of the plant cell wall. Pectin is demethylated by pectinesterase to yield pectate. Pectinesterase activity has been found in many plant tissues.
We have recently reported a 64 kDa protein from birch pollen identified as a pectinesterase and recognized specifically by IgE of a subset of birch pollen allergic patients.
The aim of the presented study was the recombinant expression and purification of the birch pollen pectinesterase in different expression systems. Domain analyses of the amino acid sequence indicated the protein as a self-regulating protein consisting of a 16 kDa regulatory domain (Pectinmethylesterase inhibitor) and a 32 kDa catalytic domain (pectinesterase). Secondary structure analysis showed that the regulatory domain is basically alpha-helikal whereas in the catalytic domain the beta-sheet structure dominates.
For recombinant protein expression a prokaryotic (E. coli) and a eukaryotic system (Insect cells) were used. In E. coli the full length protein was expressed with a N- or C-terminal HIS-tag, respectively, whereas in H5 insect cells the protein was produced without the 50 amino acid signal peptide or as 32 kDa catalytic domain fragment containing a N-terminal GST-HIS-tag. Tags were fused to simplify purification. Due to the established purification protocol using Affinity- and Size-Exclusion-Chromatography (ÄKTA-HPLC-System) recombinant proteins could be isolated in large scales at high purity grade which were recognized by specific IgE of approximately 10% of birch pollen allergic individuals. Activity in pectin-deesterification could be determined for the recombinant proteins expressed in H5 insect cells, but not for the proteins expressed in E. coli.
In conclusion the pectinesterase from birch is an enzymatically active IgE-binding protein that can contribute to understanding of crossreactivity in a subset of birch pollen allergic individuals.

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P024

Reduced response to IgE-dependent allergic reactions in IL-4 deficient mice.

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Interleukin-4 (IL-4), in addition to its pivotal role in the regulation of Th2 dominated immune responses, has previously been implicated to also contribute to the effector phase of allergic reactions. Here, we subjected IL-4 deficient mice to passive cutaneous anaphylaxis (PCA) reactions, i.e. passive sensitization by intracutaneous injection of specific IgE and systemic challenge with the corresponding allergen, to characterize the role of IL-4 in the effector phase of type-I-allergic responses. Notably, inflammatory responses to PCA as assessed by measuring ear swelling reactions following allergen challenge were found to be significantly reduced in the absence of IL-4 as compared to wild type mice (-50%, $p<0.01$). Next, we tested whether impaired PCA responses in IL-4-/- mice may be the result of altered cutaneous baseline levels of mast cells, the key effector cells in type-I-allergic responses. Histomorphometric analyses of skin sections as well as other organs (spleen, gut, peritoneum) did not yield significantly reduced mast cell populations in IL-4-/- mice. Speculating that IL-4 may contribute to allergic inflammation by facilitating the activation and/or subsequent degranulation of mast cells, we characterized mediator release in response to IgE and antigen as well as other activating signals in mast cells derived from IL-4-/- and IL-4+/+ mice. However, neither peritoneal mast cells obtained by lavage nor mast cells cultured from the bone marrow of IL-4-/- mice were found to exhibit reduced degranulation or mediator release as compared to IL-4+/+ mast cells. Taken together, our findings indicate that IL-4 contributes directly and importantly to the induction of type-I-allergic skin responses. As mast cells can reportedly release prestored IL-4 upon activation, further studies are aimed at clarifying whether these cells are the source of proinflammatory IL-4 in the effector phase of allergic reactions.

P025

Expression of RANKL, OPG, and RANK in Human Mast Cells and Bone Marrow of Patients with Mastocytosis

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Systemic mastocytosis is a clonal disease characterized by accumulations of mast cells in bone marrow and other tissues. Mastocytosis is often associated with osteopenia or osteoporosis; however, the mechanism by which mastocytosis causes osteoporosis has not been investigated to date. We therefore hypothesized that mast cells might release mediators that affect homeostasis between bone remodeling by osteoblasts and bone resorption by osteoclasts towards an increased resorption. Receptor activator of NF-κB ligand (RANKL), its receptor RANK, and its decoy receptor osteoprotegerin (OPG) are newly discovered proteins that play a key role in osteoclast differentiation and activation. Expression of RANKL, RANK, and OPG was analyzed by immunohistochemistry in bone marrow sections of 36 patients with systemic mastocytosis. In each patient, bone density was also measured. In addition, expression of RANKL, RANK, and OPG was investigated in human mast cell cultures by immunoblotting, flow cytometry, and RT-PCR. Infiltrates of mast cells in bone marrow sections stained highly positive for RANKL. Next to mast cell infiltrates, resorption lacunae containing activated osteoclasts were frequently observed. Bone and mast cell infiltrates also showed abundant expression of OPG and RANK. In vitro, expression of RANKL protein was significantly enhanced in the human mast cell line HMC-1 derived from a patient with systemic mastocytosis, compared to cultured cord blood-derived mast cells. Thus, human mast cells are able to express RANKL, RANK, and OPG, and overexpression of RANKL in mastocytosis infiltrates leading to enhanced bone resorption may contribute to the pathomechanism of osteoporosis in patients with mastocytosis.

P026

De Novo induction and Recruitment of Allergen-Specific Effector CD8+ T Cells in a Murine Model of IgE-Mediated Allergy and Airway Inflammation.

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Previous studies performed both in humans and in rodents have implicated an important, although dichotomic role of CD8+ T lymphocytes in IgE-mediated allergic inflammation. To investigate the induction and the subsequent natural course of allergen-specific CD8+ T cells, we used murine MHC class I H2Kb ovalbumin (OVA) peptide SIINFEKL (OVA257-264) tetramers and adoptive transfer with purified OVA257-264 specific CD8+ T cells from transgenic OT-1 mice. These tools facilitated to analyse the induction and the phenotype of allergen-specific CD8+ T cells in a C57BL/6 model of OVA-induced IgE-mediated allergy and airway inflammation. We observed up to 40% OVA257-264 specific CD8+ T cells, both in lung tissue and bronchoalveolar lavage (BAL) fluid of sensitised mice after repetitive OVA aerosol challenge. The vast majority (95-100%) of airway infiltrating CD8+/tetramer+ T cells showed an effector phenotype (CD62L-, CD69+, CD127-, CD44+, CD25+/-) together with a Tc1 cytokine pattern. Mediastinal but not paratracheal lymph nodes harboured significant numbers of allergen-specific CD8+ T cells. Consistently, a high number (10-30%) of allergen-specific CD8+ T cells was found in the liver even after aerosol challenge, whereas only a low number was present in the spleen during all phases of allergen exposure. Transfer of naïve allergen-specific CD8+ T cells from OT-1 mice to non-sensitised C57BL/6 hosts showed migration to the lungs and bronchoalveolar space as well as specific proliferation after OVA aerosol challenge. Thus, the use of tetramers offers for the first time the possibility to track allergen-specific CD8+ T cells directly in the natural course of IgE-mediated allergy and airway inflammation, thus avoiding a possible bias during in vitro restimulation. Our data suggest an important role for effector CD8+ T cells in the development of airway inflammation, which may be due to their specific cytokine and migration pattern.

P027

The basophil activation test based on CD63 expression in patients with immediate-type reactions to betalactam antibiotics

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Background: In vitro diagnosis of immediate type allergy to betalactam antibiotics by measurement of specific IgE has a low sensitivity. Basophil activation is associated with CD63 expression. The CD63 based basophil activation test (BAT) has proved to be very sensitive diagnostic tool in different fields of allergy.
Methods: 30 patients with a history of immediate type allergy to betalactam antibiotics and 10 controls were included in the study. We performed skin prick tests with minor determinant mixture (MDM), benzylpolylysine (PPL), penicillin, ampicillin, and amoxicillin. Specific IgE was determined by the CAP method and the BAT was performed using two monoclonal antibodies against IgE and CD63.
Results: Sensitivity and specificity of skin testing was 60 % and 100 %. Sensitivity and specificity of specific IgE and the BAT was 40 % and 100 % versus 50 % and 100 %.
Conclusions: Although both specific IgE and the BAT have a rather low sensitivity a combination of both in vitro tests raises the diagnostic yield.

P028

The CD63 based basophil activation test in patients with immediate type reactions to NSAID

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Background: To date there is no in vitro test which can reliably confirm hypersensitivity to non-steroidal anti-inflammatory drugs (NSAID). Therefore, challenge testing remains the only way of confirming intolerance of NSAID. It has been speculated that the CD63 based basophil activation test (BAT) is also helpful in elucidating non IgE-mediated immediate type reactions.
Methods: 30 patients with a strong history of immediate type reactions to NSAID and 20 controls were included in this study. We performed skin testing with aspirin, metamizol, paracetamol, diclofenac and ibuprofen and the CD63 based BAT using two monoclonal antibodies against CD63 and IgE.
Results: There was no positive skin test with any NSAID. The BAT was positive in 12/30 patients with a positive history giving a sensitivity of only 40 %, but a high specificity of 100 %.
Conclusions: The rather low diagnostic yield of the BAT performed with NSAID does not suggest that this in vitro test is able to render cumbersome challenge testing unnecessary.

P029

A Murine Model to Study the T Helper Cell Polarizing Capacity of Pollen-associated Lipid Mediators (PALMs) during Primary Sensitization In Vivo

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Pollen grains not only function as allergen carriers, but also release bioactive lipid mediators (PALMs) upon contact with the aqueous phase. PALMs modulate human dendritic cells function in a fashion that results in a Th2 polarization of human naïve T cells in vitro. To address, whether PALMs show similar effects in vivo, we analyzed events of early T cell polarization during primary sensitization in mice. To overcome the low frequency of naïve antigen-specific T cells in wild type mice, we established a DO11.10/BALB/c chimera using adoptive transfer, thus ensuring a defined number of OVA323-339-specific CD4 cells in regional lymph nodes. 48 hours after intranasal instillation of OVA323-339 alone or OVA323-339 plus aqueous birch pollen extract (APE) to DO11.10/BALB/c chimeras, cells were obtained from draining lymph nodes, restimulated with peptide in vitro and on day 6 intracellular IL-4 and IFN-γ were analyzed by flow cytometry. Transfer of 5x106 T cells led to 1-2% of OVA323-339 TCR transgenic T Cells in regional lymph nodes. 48 hours after intranasal instillation of OVA323-339, proliferation of antigen specific T cells was detected in draining, but not in non-draining lymph nodes. In comparison to exposure with OVA323-339 alone, intranasal instillation of OVA323-339 plus APE lead to an increase in IL-4 - and a decrease of IFN-γ producing antigen specific T cells, as determined after peptide restimulation. In conclusion, PALMs appear to exert immunomodulatory activities on the early phase of primary sensitization in vivo, that result in a bias towards Th2 polarization.

P030

Selective photothermolysis of blood vessels following FPDL-treatment in vivo

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Laser therapy is the standard treatment for vascular lesions like port-wine stains (PWS). The flashlamp-pumped pulsed dye laser (FPDL) (wavelength: 585 nm; pulse duration: 0.45 ms) is mainly used. However, successful treatment relies on the laser light absorbed by the endogenous chromophore haemoglobin and is unsatisfying for many lesions. The goal of this work is to investigate the effects of the FPDL on blood vessels regarding vessel diameter in vivo and to correlate the experimental results with the predictions of a mathematical model.
The dorsal skinfold chamber model in hamsters (n = 18) was used for monitoring the vascular effects of FPDL treatment (λ=585 nm; pulse duration: 0.45 ms; fluence: 6 J/cm²; Candela Corp., Wayland, MA, USA). Diameters of vessels (n = 3394; ranging form 2 to 186 μm) marked with FITC-dextran (MW 150 000) were measured using intravital fluorescence microscopy prior to and 15 min, 1 h and 24 h following irradiation. Histology was taken 1 h and 24 h after therapy and tissue sections were stained with H&E, TUNEL or CD31. The in vivo results were correlated with the predictions of a mathematical model based on the finite element method (FEM).
FPDL treatment revealed a less pronounced effect on smaller blood vessels as the number of unperfused vessels increases with increasing diameter. Overall 87.1 % of the vessels with a diameter ranging from 30 to 100 μm were unperfused at 15 min following FPDL treatment lasting for 24 h. The correlation of the observed with the calculated reduction of perfused vessels was in agreement as the corresponding graphs showed a similar distribution. Histology indicated that 1 hour following treatment thermal damage induced immediate coagulation restricted to the irradiated area, whereas at 24 hours a higher degree of tissue damage was observed. This indicates a delayed biologic response contributing to the extent of laser-tissue interaction.
The experimental results obtained in this model can be described by a mathematical model. The lack of efficacy regarding the destruction of smaller vessels (2-25 μm) may explain the lack of complete PWS blanching response in the clinical setting. According to the experimental model this is very likley due to the low hematocrit in these vessels.

P031

A safe and efficient dominant-negative HSV-1 viral vector for gene delivery in vitro and in vivo

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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 large genome size, 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. Several critical technical barriers have prevented the development of effective and safe herpes viral vectors, including: 1.) assurance that the viral vector is incapable of self-replication 2.) preventing recombination in the context of wild-type HSV-1, and 3.) providing lasting therapeutic effects in the absence of self-replication. This study demonstrates the development of a unique new herpes-simplex virus 1 (HSV-1) based vector that uniquely fulfills these requirements.

We generated an HSV-1 recombinant CJ83193, by selectively regulating the expression of the trans-dominant-negative mutant polypeptide, UL9-C535C, of HSV-1 origin binding protein UL9 with the tetracycline repressor (tetR)-mediated gene switch. 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 demonstrate the infection efficiency and cell cytotoxicity of CJ9-LacZ in vitro and demonstrate that CJ9-LacZ can serve can serve as a highly effective gene transfer vehicle to fibroblasts, keratinocytes, Vero and osteosarcoma cells in vitro. Furthermore we demonstrate that microseeding of CJ9-lacZ into a mouse footpad leads to gene expression in various cell types in vivo and brain injection leads to high expression of ß-Galactosidase in brain tissue.
The results provide initial demonstration of a safe and effective HSV viral vector that encodes a unique safety mechanism capable of inhibiting its own replication and replication of wild-type virus in the host.

P032

Correlation between pro-inflammtory cytokines and concentration of IgA antibodies of chlamydia trachomatis in seminal plasma of men

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Chlamydia trachomatis causes widespread, often subclinical genital tract infection in both men and women, but its relevance for male infertility is still under debate.
The aim of this study was to determine the correlation between positive/negative seminal plasma IgA and the concentration of interleukin 6 (IL 6), interleukin 8 (IL 8), interleukin 10 (IL 10), interferon gamma (INF-γ) and tumour necrosis factor (TNF-α). The presence of these cytokines was investigated in seminal plasma of 72 patients divided as follows: into 3 groups according to their main disease ((1) patients with unspecific genital tract infections, (2) patients with haematological malignancies and (3) patients with testicular tumours) and within each of these 3 groups into 2 subgroups according to a positive or negative seminal plasma IgA, using specific enzyme-linked immunosorbent assays, to study their role in male infertility (Distribution by Biozol,Germany). Statistical analyses were performed using t-test.
There were no differences in IL 6, IL 10 and TNF-α concentrations between the subgroups ($p>0,05$). IL 8 was present in higher levels in the seminal plasma of patients with a positive seminal plasma IgA in group: (1):($p=0,006$).
INF-γ was present in significantly higher levels ($p=0,026$) in the seminal plasma of patients of group (2) with a positive seminal plasma IgA ($3\pm1,53$pg/ml) compared to patients of group (2) with a negative seminal plasma IgA ($0,99\pm1,39$pg/ml).
These data suggest that IL 8 might be used as sensitive marker for chlamydial genital tract infection in patients with testicular tumours and that INF-γ might be used as a sensitive marker for chlamydial genital tract infection in patients with haematological malignancies.

P033

Pharmacological manipulation of the nude phenotype: Cyclosporin A partially compensates for non-functional Foxn1 via modulation of hair matrix and dermal papilla functions

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In nude mice which have structural hair shaft abnormalities due to the lack of functional Foxn1 transcription factor, cyclosporine A (CsA) induces hair growth. It is still unknown how hair shaft formation and hair follicle cycling are affected by CsA treatment of Foxn1nu/nu mice. Therefore, we examined whether and how CsA alters important hair research parameters. We show that CsA (30 mg/kg/d i.p.) promotes anagen development and hair shaft elongation in vivo and in vitro in Foxn1nu/nu mice, but fails to inhibit spontaneous catagen development, and does not alter the expression of a key hair keratin whose transcription is controlled by Foxn1 (mHa3). CsA also increases hair matrix keratinocyte proliferation, the number and proliferation rate of dermal papilla fibroblasts, and the dermal papilla immunoreactivity for insulin-like growth factor receptor-1 in Foxn1nu/nu mice. Given that the hair growth-modulatory effects of CsA may be related to its inhibitory effects on protein kinase C (PKC) signaling, it is interesting to note that CsA indeed decreased the levels of PKC-isoforms a and ß, and increased immunoreactivity for PKC- e and z. Therefore, the induction of hair gowth in nude mice by CsA primarily does not reflect effects on hair keratinocyte differentiation, but is largely due to anagen induction and the stimulation of hair matrix keratinocyte proliferation and dermal papilla functions, mediated perhaps in part by changes in local PKC signaling.

P034

Role of sensory nerves and mast cells in cutaneous inflammation induced by the vanilloid receptor ligand capsaicin.

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The proinflammatory properties of capsaicin (CPS), the pungent principle of pepper, are generally contributed to its activating effects on sensory nerves, as CPS is known to act via vanilloid receptors (VR) and to induce VR-mediated release of neuropeptides from sensory nerves. However, VR have recently been shown to also be expressed by various epithelial and mesenchymal cell types including mast cells. To better characterize the cellular mechanisms of cutaneous responses to CPS, we investigated CPS induced skin inflammatory reactions in murine skin that lacked sensory nerves or mast cells. As expected, we found that skin inflammatory responses to CPS as assessed by measuring ear swelling reactions were markedly reduced (-90%, $p<0.001$) in C57BL/6 mice that had been subjected to surgical unilateral ablation of the cutaneous nerves of the ear. Morphometric analyses of denervated CPS treated ears revealed unaltered mast cell numbers and reduced rates of mast cell degranulation. Surprisingly, skin inflammation induced by CPS was also reduced in genetically mast cell deficient KitW/KitW-v mice, albeit to a lesser extent (-50%, $p<0.01$), indicating that mast cells contribute to the induction of sensory nerve dependent CPS induced skin inflammation. Our data demonstrate that CPS elicits cutaneous inflammatory responses by acting directly on sensory nerves, most likely via VR, and that normal sensory nerve dependent inflammation involves and requires the activation of skin mast cells. Our findings suggest, but do not prove, that mast cells, in the context of CPS induced inflammation, degranulate in response to neurogenic signals.

P035

A novel "microsphere" in vitro-assay system for the imitation and pharmacological manipulation of basic epithelial-mesenchymal interactions in the human hair follicle

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The meaningful study of human hair growth in vitro currently is only possible with organ-cultured human scalp hair follicles (hHF) in anagen VI. However, simplified organotypic systems are needed for the experimental dissection of critical epithelial-mesenchymal interactions that underlie human hair growth. Our aim was to develop a novel, easily handled in vitro-assay which meets all the basic criteria for organotypic systems that wish to imitate essential hHF epithelial-mesenchymal interactions and to investigate, in this assay, the effect of selected agents that are known to modulate hHF growth in vivo. Organotypic cultures of co-cultured hHF ORS keratinocytes (ORSK) and hHF dermal papilla fibroblasts (DPC) in a matrix-mixture of collagen I and Matrigel™ were established, using a novel methodology for generating "microspheres". These were cultured submerged in media with different calcium concentrations and appropriate test agents and their morphological and functional characteristics were then assessed by routine histology, histomorphometry and immunohistochemistry. Our results showed that ORSK formed concentric aggregates in these microspheres which vaguely resembled primitive ORS structures and expressed ORS markers (CK6, CK14, TGFβ2 and IGF-I receptor), while the DPC expressed the hair marker versican. In these microspheres, the hair growth-inhibitory agents, tretinoin and TGFβ2 inhibited the proliferation of ORSK aggregates, and also induced ORSK apoptosis, while CRH inhibited the proliferation of ORSK and DPC. IGF-I, a key growth promoter for hHF,stimulated the proliferation of both ORSK and DPC. The regulation of proliferation by cyclosporine A and HGF was found dependent on Ca2+ concentration in medium. In addition, certain hair-related markers (p75NTR, β-catenin, SCF) were up-regulated in some of the groups treated with recognized hair growth-modulatory agents. This new microsphere system allows the standardized preclinical assessment of test agent effects on proliferation, apoptosis and key marker expression of human ORSK and DPC under substantially simplified in vitro-conditions which approximate the in vivo-situation.

P036

Systembiologisches Modell zur computerbasierten Simulation dynamischer Prozeße in der Epidermis

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Obwohl die Epidermis ein relativ einfach aufgebautes Gewebe darstellt und zu über 90% aus einer Zellpopulation, den Keratinozyten, besteht, sind die dynamischen Entwicklungsprozesse von den Stammzellen bis zum Korneozyten und die Zell-Zell Interaktionen äußerst komplex. Die systembiologische Modellierung biologischer Vorgänge beschränkt sich bisher hauptsächlich auf intrazelluläre Vorgänge, d.h. das Genom und das Proteom. Wir haben ein agentenbasiertes Computermodell entwickelt, das es erstmals erlaubt die komplexen dynamischen Abläufe epidermalen Wachstums zweidimensional zu simulieren. Das Modell besteht aus Softwareagenten, die Stammzelleigenschaften besitzen und solchen, die über die Eigenschaften von transitorisch amplifizierenden Zellen verfügen; weiterhin sind Zellen enthalten, die ausdifferenzieren können. Mit Hilfe des Modells gelingt es, ein geschichtetes Plattenepithel zu simulieren, das histologisch validiert werden kann. Das Computermodell enthält zusätzlich Informationen über die Polarität der epithelialen Zellen. Störungen der Polarität spielen wahrscheinlich eine wichtige Rolle für die Entstehung von Karzinomen. Das vorgestellte Modell könnte geeignet sein, die komplexen Interaktionen zwischen Tumorzellen und umgebenden Gewebe zu simulieren und dadurch besser zu verstehen.

P037

Translational research from Dermatology to Cardiology: Characterization of a novel anti-inflammatory peptide

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We characterize a novel anti-inflammatory function of the fibrin-derived peptide, Bbeta15-42. This peptide competes with the fibrin E1 fragment analogue NDSK-II for binding to endothelial VE-cadherin, thereby preventing transmigration of T cells, monocytes and neutrophils across endothelial cell monolayers. Employing a model of regional ischemia/reperfusion in the isolated rat heart, we show that peptide Bbeta15-42 reduces infarct size, when hearts are perfused with freshly drawn blood, but not in hearts perfused with plasma or blood depleted of white blood cells. Also in acute and chronic in vivo rat models of ischemia/reperfusion injury, the peptide significantly reduces leukocyte infiltration, infarct size and subsequent scar formation. The pathogenic role of fibrinogen progenies is further documented in ischemia/reperfusion experiments using fibrinogen-/- mice, where infarcts are significantly smaller and Bbeta15-42 has no cardioprotective effects. This leads to the concept that the interplay of fibrin fragments, white blood cells and endothelial VE-cadherin contributes to the pathophysiology of myocardial damage following reperfusion. The peptide Bbeta15-42 represents a potential candidate for reperfusion therapy in humans.

P038

Hyperthermia inhibits proliferation, promotes apoptosis and attenuates mediator release in mast cells

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Hyperthermia in combination with various chemotherapeutics is one possible strategy combating destined tumors. Depending on the intensity and duration of application, hyperthermia induces necrosis or apoptosis in the targeted tissue. Cutaneous mastocytoma (CM) is a benign, tumorous disease characterized by enhanced proliferation and subsequent accumulation of cutaneous mast cells (Mc) in the dermis. We have recent evidence that whole body hyperthermia or local hyperthermia achieved by infrared A (IrA) irradiation attenuates the progression of CM. This study was aimed to investigate the effect of hyperthermia on Mc proliferation and apoptosis in vitro using HMC-1. Moreover, impact of hyperthermia on Mc degranulation was determined by using LAD-2 and primary Mc. For proliferation studies HMC-1 were kept at 42°C up to 120 minutes, followed by recultivation at 37°C for variable intervals. Viability of HMC-1 was determined using a WST-1 assay and cell number was checked in a casy1 cell counter. Apoptosis was monitored by using a caspase 3 activity assay kit. For degranulation assays, LAD-2 and Mc were loaded with human IgE 24 hours prior to thermal treatment. Afterwards cells were kept either at 42°C up to 2 hours to test the effect of hyperthermia, or were irradiated with an water filtered IrA source. In the latter case cells were tempered to 37°C thus enabling to test for the effect of irradiation alone. Mc degranulation was induced by IgE-receptor crosslinking, and histamine release measured as an indicator of Mc activation. Heat treatment of HMC-1 decreased proliferation time-dependently with a maximal reduction of 50%. Additionally hyperthermia induced apoptosis of HMC-1, peaking 12h after treatment, which is in line with data for UVB-induced apoptosis in HMC-1. Histamine release from LAD-2 and Mc was time-dependently reduced by hyperthermia. IrA irradiation alone without hyperthermia had no effect on Mc degranulation. We suppose that hyperthermia elicits several effects in Mc, namely antiproliferation, induction of apoptosis and inhibition of mediator release which may all be beneficial for the treatment of CM. Further investigations are necessary to elucidate the cellular mechanism underlying these hyperthermia-effects, which may be the basis for future approaches for the treatment of Mc mediated disorders.

P039

Expression-pattern of mast cell phenotypes in cutaneous tumours

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The expression of mast cells is increased in the vicinity of various tumors. It is not known, however, which type of mast cell is preferably overexpressed: mast cells containing merely chymase (MC), mast cells containing only tryptase (MT), or mast cells expressing both proteases (MCT). In order to address this question, human melanomas, spinaliomas and basaliomas of various stages were cut into sections of 5 μm after therapeutical excision. Sections were forwarded to immunohistochemical double-stainings or immunofluorescent co-localisation studies, respectively, using antibodies directed against chymase, tryptase and c-kit. Additional toluidine-blue stainings were performed for the detection of mast cells. In melanomas and spinaliomas, no increase in mast cell numbers was observed as long as the tumors could be classified as being "in-situ". However, as soon as the tumors began to grow invasively, an increase in surrounding fibrous tissue coinciding with an increase in mast cells was observed. In basaliomas, an increase in mast cells within the surrounding tissue could be observed in all cases examined. In all sections, no matter which tumour, tryptase- plus chymase-containing mast cells (MCT) accounted by far for the majority of the mast cell population as they also did in healthy controls. The proportion of exclusively tryptase-containing mast cells was slighty increased in tumour-surrounding fibrosis (max. 10%), but in general, the proportion of mast cell phenotypes (MCT : MC : MT) mirrored the conditions found in normal skin. From these data we conclude, that mast cells are increased in the tumor-surrounding fibrous tissue as soon as the tumor gets invasive by penetrating through the basal membrane, but that the proportion of mast cell phenotypes stays basically unchanged.

P040

IL-22 increases the innate immunity of the skin

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Interleukin(IL)-22 was discovered in 2000. It belongs to the IL-10 family of cytokines whose members (additionally IL-10, IL-19, IL-20, IL-24, and IL-26) are structurally related molecules. We have previously shown that IL-22 is mainly produced by activated T cells, particularly the Th1 subset. Unlike IL-10, the data presented here surprisingly show that IL-22 does not act on immune cells. This conclusion is based on a systematic study at all possible levels of analysis: on receptor expression, signal transduction, effects in vitro, and effects in vivo. In contrast, the quantitative analyses of a wide range of tissues and corresponding primary cells and cell lines showed that many non-immune tissue cells are target of IL-22 as they express both chains of the IL-22 receptor complex. Very high levels of IL-22 receptor chains were found in skin and keratinocytes. In primary human keratinocytes these levels were further upregulated by IFN-γ suggesting an increased sensitivity of these cells towards the IL-22 action under T1 conditions. The receptor complex on these cells was functional as deduced from IL-22-induced STAT3 tyrosine phosphorylation. For the first time, this study also identified effects of IL-22 on keratinocytes, namely the upregulation of the antimicrobial agents β-defensin 2 and β-defensin 3. This effect was transcriptionally regulated, and independent on protein de novo synthesis and alternative protein secretion indicating a direct effect of IL-22. Additionally, this induction was time- and dose-dependent, and enhanced upon cellular differentiation. The extent of induction was comparable to that by other known inducers of β-defensins. In skin from patients with T cell-mediated dermatoses, high levels of IL-22 were highly significantly associated with strongly upregulated expression of β-defensin-2 and β-defensin-3 suggesting a protective effect of IL-22 in these disorders. Taken together, IL-22 does not serve the communication between immune cells but is a T cell mediator that directly promotes the innate, non-specific immunity of the skin. The observation that activated T1 cells directly regulate the non-specific immune defense in tissues demonstrates a so far unknown but very important side of the immune system.

P041

Is there an interaction between interleukin-10 and interleukin-22, two cytokines important for skin homeostasis?

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Interleukin(IL)-10 and IL-22 are structurally related cytokines. Their heterodimeric receptors consist of the cytokine-specific chains IL-10R1 and IL-22R1, respectively, and the common chain IL-10R2. This study focused on the question of whether IL-10 modulates IL-22 effects and vice versa. This question is important because (i) IL-10 and IL-22 exert anti- and pro-inflammatory effects, respectively, (ii) both cytokines are important for skin homeostasis, and (iii), as we show here, are simultaneously present in both systemic and local immune activation. The revealed lacking concomitance of IL-10R1 and IL-22R1 on identical cells excluded any possible interaction between IL 10 and IL-22 apart from the competition for IL-10R2. To study this competition, monocytes and hepatocytes were chosen. The dependence of the cytokine action on IL-10R2 was verified. Interestingly, no influence of IL-22 on IL-10 effects was observed. The same was true when IL-22 was used in complex with IL-22 binding protein. Similarly, no influence of IL-10 was found on IL-22 action. This missing competition seemed to be due to a lack of binding between IL-10R2 and the native cytokines in the absence of the corresponding R1 chain. However, IL-10R2 interacts with defined IL-10 and IL-22-derived peptides supporting the hypothesis that binding of the cytokines to the R1 chain creates a binding site for IL-10R2.

P042

IL-19, IL-20, IL-22, IL-24 and IL-26: are they interleukins?

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This study investigated the expression of five novel human IL-10-related molecules and their receptors in blood and tissue cells. IL-19 and IL-20 were found to be preferentially expressed in monocytes. IL-22 and IL-26 expression was exclusively detected in T cells, especially upon type 1 polarization, and in NK cells. IL-24 expression was restricted to monocytes and T cells. Detection of these molecules in lymphocytes was predominantly linked to cellular activation. With regards to T cells, IL-26 was primarily produced by memory cells, and its expression was independent of co-stimulation. In vivo, the novel molecules were expressed preferentially within inflamed tissues. However, they cannot act on immune cells due to the lack of corresponding receptor chains in these cells. Instead, several non-immune tissues, particularly the skin, the reproductive and respiratory systems, and various glands appear to be the main targets of these cytokines. Interestingly, in keratinocytes, the main cells of the skin epidermis, these receptors are specifically regulated under T1 and T2 condition. Taken together, immune cells may represent a major source but a minor target of the novel IL-10 family members, but non-immune tissues, especially the skin, are major targets of these mediators.

P043

Expression of melanoma associated human endogenous retrovirus is stimulated by ultraviolet radiation

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Human endogenous retroviruses (HERV) represent a cellular reservoir of potentially pathogenic retroviral genes. During development of melanoma the production of retroviral particles is activated. In this study we investigated the effect of ultraviolet radiation (UVR) on expression of melanoma associated endogenous retrovirus (MERV) in different melanoma cell lines. Treatment of different melanoma cells with UVB (200 mJ/qcm) resulted in approximately 10-fold increase of retroviral particles in the supernatants. Expression of the corresponding retroviral proteins was also significantly increased. In addition, transcription of the retroviral prot-gene, but not of the cellular beta-actin gene was several magnitudes higher upon treatment of the cells with UV-light. The data indicate that expression of MERV can be activated by UV-light.

P044

The expression of Nerve Growth Factor and its precursor proNGF together with their high affinity receptors TrkA and p75 respectively suggest distinct growth factor properties in defined epithelial compartments of the murine skin.

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The prototypic nerve growth factor (NGF) promotes neuronal survival but also keratinocyte proliferation via its high affinity receptor tyrosine kinase A (TrkA) and was suggested as a hair growth promotor. By contrast, the NGF precursor proNGF was recently shown to be a high affinity ligand for p75, involved in keratinocyte apoptosis and hair follicle regression. We found, that pro-NGF/NGF mRNA levels peaked in early anagen, dramatically decreased towards high anagen and recovered in catagen while pro-NGF/NGF protein peaked in catagen. Immunohistochemistry revealed strongest NGF and TrkA expression in the proliferative basal layer of the epidermis and distal outer root sheath throughout the hair cycle. In contrast, strong proNGF and p75 expression was found in the distal epidermal layers and in the highly differentiated inner root sheath throughout the cycle and in the epithelial sack of catagen hair follicles. Adjacent to the proNGF+ hair follicle epithelium, p75 was also detected in the dermal papilla of late anagen hair follicles prior to the onset of catagen and in the regressing epithelial strand in catagen. These distribution patterns suggest a role for NGF/TrkA interactions in epidermal and follicular keratinocyte proliferation and hair growth as well as a role for proNGF/p75 interactions in terminal keratinocyte differentiation and hair follicle regression. Accordingly, we found in organ culture that a standard commercial NGF-preparation containing proNGF and NGF promoted anagen in early anagen hair follicles, while it induced catagen in late anagen hair follicles. Live and death decisions in NGF-treated tissues thus depend on the availability of the respective receptors. The development of NGF-preparations exclusively containing NGF or proNGF may provide a promising strategy for the development of new therapeutic approaches to treat hair growth disorders and wound healing.

P045

The Endogenous Angiogenesis Inhibitor Thrombospondin 1 is an Anti-inflammatory Agent in Allergic Contact Dermatitis

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Recent data have shown that inhibition of angiogenesis leads to a decreased inflammatory response in experimental contact dermatitis. Thrombospondin 1 (TSP-1) is a potent endogenous angiogenesis inhibitor, which is supposed to be critically involved in the maintenance of cutaneous vascular quiescence. Furthermore TSP-1 protein and mRNA are upmodulated in the inflamed lesions of patients undergoing patch tests when compared to normal skin taken from the same patient. We induced contact hypersensitivity reactions by topical application of oxazolone to the skin of mice with targeted epidermal TSP-1 overexpression, in TSP-1-deficient mice and in FVB wildtype mice. When we studied the timecourse of TSP-1 modulation in FVB mice, we found a peak in TSP-1 expression 24 hours after the onset of an inflammation with a gradual return to baseline levels by 96 hours, mirroring the expression pattern of pro-angiogenic factors such as Vascular Endothelial Growth Factor A (VEGF-A), Placental Growth Factor and Interleukin 8. In addition, we observed decreased edema formation in the inflamed ears of TSP-1 transgenic mice associated with a significant decrease in the number of enlarged blood vessels when compared to the FVB littermates. Conversely, TSP-1-deficient mice exhibited a persistent reaction, characterized by a delayed resolution of the inflammation and enhanced vascular remodeling, when compared to wildtype mice. Moreover, histological analyses of the inflammatory infiltrate using specific antibodies revealed a reduced number of macrophages in the TSP-1 overexpressing mice compared to the wildtype littermates. Reduced edema formation in the transgenic animals resulted from lower plasma leakage as demonstrated by an Evans blue leakage assay revealing 45% less leakage in the transgenic mice when compared to the control littermates. In a modified Miles assay, TSP-1 overexpression resulted in reduced VEGF-induced permeability implying that the mechanism responsible for the observed effect may involve, at least in part, an inhibition of VEGF-mediated hyperpermeability. These data suggest that TSP-1 is an anti-inflammatory factor helping in the resolution of an allergic contact dermatitis. Currently, several different approaches designed to increase the levels of TSP-1 are being developed for the treatment of cancer. Our data indicate that such therapies may also be beneficial for the treatment of inflammatory diseases.

P046

The Angiogenesis Inhibitor Vasostatin Suppresses the Effector Phase of Allergic Contact Dermatitis.

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Endothelial cells are critically involved in the pathogenesis of allergic contact dermatitis since angiogenesis and increased vascular leakage are characteristic features of acute inflammatory processes. Therefore, administration of angiogenesis inhibitors might exert a beneficial effect. The present study was performed to investigate the effect of Vasostatin on the manifestation of contact hypersensitivity (CHS) responses induced by haptens. Vasostatin is an endothelial cell inhibitory factor isolated from Epstein-Barr virus- immortalized cells and was identified as the amino terminal domain of Calreticulin (amino acids 1-180). Vasostatin inhibits the proliferation of endothelial cells and suppresses neovascularization and tumor growth in vivo. To study the effect of Vasostatin on CHS, FVB mice were sensitized against oxazolone. Five days later, ears were challenged with oxazolone and CHS responses were measured. Mice that received i.p. injections of Vasostatin revealed a significantly reduced ear swelling response in comparison to mice treated with saline. These differences were observed throughout the entire recovery period of 7 days. Histological analysis revealed reduced edema and a significant decrease in blood vessel density in the inflamed ears of the Vasostatin-treated mice. Reduced edema formation resulted from lower plasma leakage in Vasostatin-treated mice, as demonstrated by Evans blue leakage assays. Vessel spread analysis combined with three-dimensional vessel imaging using whole mounts of ears following lectin perfusions demonstrated fewer larger vessels in the treated group compared to the control animals. The observed decreased number of adherent leukocytes in the lectin perfused ears of treated animals is in accordance with intravital microscopy studies showing a significant decrease in the rolling fraction of leukocytes in Vasostatin-treated mice. Furthermore, histological analyses of the inflammatory infiltrate using specific antibodies revealed a reduced number of neutrophils and macrophages in the treated ears compared to the untreated mice. Electron microscopy studies indicated that Vasostatin might prevent leakage by maintaining the integrity of interendothelial junctions. This observation was confirmed when we observed an inhibition of VEGF-induced VE-cadherin reorganization in cultured human dermal microvascular endothelial cells following Vasostatin treatment. Furthermore, in a modified miles assay, Vasostatin potently inhibited VEGF-induced permeability in-vivo implying that the mechanism responsible for the observed effect may involve, at least in part, an inhibition of VEGF-mediated hyperpermeability. The present data suggest that 1) angiogenesis may play an important role in the elicitation phase of CHS and 2) that angiogenesis inhibitors like Vasostatin are potential candidates for the treatment of inflammatory dermatoses including allergic contact dermatitis.

P047

Keratin5-Cre/LoxP mediated deletion of Vascular Endothelial Growth Factor reduces overall fertility of female mice and compromises mammary gland function by reducing vascularisation during pregnancy and lactation.

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During pregnancy and lactation, the mammary gland ductal system, an appendage of the skin, undergoes expansion and differentiation to form a system of highly metabolically active, milk secreting alveoli. This process requires angiogenesis, and Vascular Endothelial Growth Factor (VEGF), a major blood vessel mitogen and permeability factor, is upregulated during mammary gland differentiation. We have previously reported that inactivation of VEGF in mammary gland epithelial cells leads to reduced milk production, retarded ductal development and stunted pup growth. Analysis of the milk has now shown no difference in total protein content, nor in the milk protein, casein. Copious VEGF could be detected in milk from control mice, but was completely absent in mutant milk, and an accumulation of lipid droplets in the lactating glands suggests an impairment of secretory function. CD31 staining to identify blood vessels revealed a significant reduction in area occupied by the vasculature in the glands of mutant mice compared to controls, in association with decreased proliferation and increased apoptosis of blood vessel endothelial cells. We conclude that VEGF, secreted by mammary gland epithelial cells, contributes significantly to expansion of the vasculature during pregnancy and lactation, thereby supporting mammary gland duct growth. In addition to inadequate mammary gland function, these mutant females display a severe defect in fertility, displayed by difficulty in conception, loss of embryos during pregnancy, and a reduced number of live births. Since, in our mouse model, VEGF is also deleted in the uterine epithelium, this cytokine probably also plays an important role in the development of the vasculature of the uterus during pregnancy.

P048

Melanin contamination in DNA/RNA preparations from melanoma samples inhibits PCR: how to get rid of it !

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The isolation of DNA or RNA from tumor material and subsequent PCR is a frequently performed method. Melanoma tumor samples, however, are often rich in melanin which co-purifies with DNA and RNA and inhibits thermostable DNA-polymerase during PCR. Melanin is not a single molecule, but a mixture of highly polymeric non linear structures consisting of up to several hundred monomeric units. Different methods have been proposed to remove melanin, but they are laborious and do not always lead to satisfying results.
We have established a flexible and relatively easy protocol to drastically reduce melanin contamination in RNA preparations. Since melanin absorbs UV-light also at 260 nm, the polynucleotide concentration in melanin containing samples cannot be determined with the standard photometric measurement. We therefore generated a photometric protocol to determine both, the melanin- and the RNA concentration in our samples. Using artificial melanin, we determined the absorption coefficient of melanin at 320 nm and at 260 nm. This allowed us to measure the melanin-concentration in any given sample and the RNA-concentration could then be correctly calculated from the samples absorption at 260 nm. Subsequently, different combinations of RNA-purification-methods were compared regarding efficacy of melanin removal and yield of RNA. Using the best protocol, a combination of RNA extraction with Qiagen RNAeasy kits followed by repeated purification over ion-exchange columns, we were able to remove more than 97% of the melanin while retaining 66% of the RNA in samples obtained from highly pigmented melanomas.. The capability of the different RNA preparations to function as template in an RT-PCR was tested and inversely correlated to the melanin contamination. Our RNA purification method will help all scientists who are interested in isolation of polynucleotides from melanin-containing tumor samples.

P049

Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide production

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Tolerogenic activity of dendritic cells (DC) has so far mostly been attributed to immature or semi-mature differentiation stages but never to their precursor cells. Here, we describe the generation of myeloid DC precursors with potent suppressive activity on T cell responses.
These myeloid suppressor cells (MSC) appear after culture of bone marrow (BM) cells for 8-10 days under low GM-CSF or early after 3-4 days under high GM-CSF conditions. They represent early myeloid precursor cells with ring-shaped nuclei expressing Gr-1low (i.e. Ly-6C+, Ly-6Glow) CD11b+ CD31+ ER-MP58+ asialoGM1+ F4/80+. MSC are not the earliest hematopoietic stem cells (CD34-), DC (CD11c-), granulocytes (Gr-1high), T or B cells or plasmacytoid DC (CD4-, CD8- B220-), or NK/NKT cells (DX5- NK1.1-) but represent pre-DC as sorted MSC develop into CD11c+ DC within 6 days. Suppressor activity partially depends on IFN- stimulation and is mediated through mechanisms
requiring cell contact and nitric oxide but is independent of TNF, CD1d and TGF-. Injection of MSC into allogeneic mice abrogated the proliferation of their spleen cells upon allogeneic restimulation ex vivo, indicating that these MSC act suppressive also in vivo.

P050

EFFECTS OF THE DUAL ENDOTHELIN RECEPTOR ANTAGONIST BOSENTAN IN PATIENTS WITH SEVERE SECONDARY RAYNAUD#S SYNDROME

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Endothelin, a naturally occurring peptide has essential developmental and regulatory roles in vessel physiology. However, it is also a pathogenetic mediator with a number of deterious effects, including vasoconstriction, fibrosis, vascular hypertrophy and inflammation. Overexpression of endothelin-1 (ET-1) has been shown to be associated with various systemic diseases including pulmonary arterial hypertension (PAH), systemic scleroderma and systemic lupus erythematosus. Raynaud#s phenomenon, secondary to collagen vascular diseases, is a common problem which is troublesome and frequently difficult to treat. Elevated ET-1 serum concentrations have been shown to be associated with the duration of vasospasms in this syndrome.

Bosentan is an orally active nonpeptide antagonist of endothelin receptors and has been approved for the treatment of PAH. Moreover, recent studies have demonstrated benefitial effects of Bosentan in patients with scleroderma, leading to a reduction of newly developed digital ulcerations.
Here we report four patients with a long history of severe secondary Raynaud#s Syndrome without digital ulcerations, who have been treated with Bosentan 125mg BID for a 16 weeks period during winter season. Clinical parameters such as number, severity and duration of daily attacks (finger discoloration and pain severity) and quality of life asessment (Raynaud#s condition score) were regularly examined. In addition objective parameters, including digital arterial oscilography, digital arterial blood pressure under various temperature conditions (room temperature, 20°C, 40°C), capillary microscopy and rewarming time were asessed before and after the treatment period.
In all patients, 3 months of treatment with 2x 125mg Bosentan resulted in a clear improvement of symptoms associated with their disease: numbner of Raynaud attacks, pain severity, Raynaud#s condition score and subject#s discomfort.
These findings are further supported by improved rheologic capillary parameters. Thus, we conclude that blockade of ET-1 with Bosentan represents an effective therapeutic option in patients with secondary Raynaud#s Syndrome.

P051

Inhibitory effects of dimethylfumarate on angiogenesis in vitro

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Psoriasis is a chronic inflammatory autoimmune disease of skin and small joints, that results in a severe impairment of quality of life. A characteristic feature of psoriasis of the skin is the formation of erythematous plaques, infiltrated by interferon-γ producing. T cells, mast cells and neutrophils. Keratinocytes of psoriatic lesions show an increase in Vascular Endothelial Growth Factor (VEGF) expression, whereas its high affinity receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) are upregulated on the microvascular endothelial cells. The expansion of the microvasculature is accompanied by an increase in proliferation and $\alpha v\beta 3$ expression of endothelial cells. This profound angiogenic activity of the superficial microvasculature of lesional skin suggests a crucial role for angiogenesis in the pathogenesis of psoriasis. Here, we analyzed the influence of the potent anti-psoriatic agent Dimethylfumarate (DMF) on angiogenesis. In the chorioallantoic membrane (CAM) assay DMF led to changes in blood vessel morphology and embryonic lethality. To investigate the effects of DMF on endothelial cells in more detail, we performed the 3-dimensional spheroid based angiogenesis assay and a second in vitro assays of sprouting angiogenesis. In both assays, DMF induced a strong inhibition of sprout formation in a dose-dependent manner. In addition, endothelial cell migration was strongly decreased by the influence of DMF. These results suggest, that the anti-psoriatic activity of DMF is, at least in part, a result of its anti-angiogenic potential.

P052

TTGE is most appropiate for PCR diagnosis of cutaneous T-cell lymphoma

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Aim: In recent years clonality analysis has been a helpful tool in diagnosis of cutaneous T-cell lymphomas, especially in early forms. The aim of this study was to modify PCR methods and to evaluate their usefullness as routine procedures.
Material: 20 patients, 16 samples of mycosis fungoides and 11 samples of pleomorphic cutaneous T-cell lymphomas investigated. compared to 10 histologically proven subacute dermatitis.
Method: The DNA from paraffin blocks was extracted and the success was proven with a PCR of the beta-globulin-gene. We used four Vg-Primer (Vg-1-11) and four Joining-Primer (Jg-1/2, Jg-p, Jg-p1 and Jg-p2). In 12 PCRs the primer combinations for the g-chain of the T-cell receptor was tested and separated on 6% polyacrylamid gel. We tested every tumor sample by using the combination of all Vg-Primers with the Joining-Primer Jg-1/2. The tumor samples showing no clonality were further analysed with additional three Joining-Primers (Jg-p, Jg-p1 and Jg-p2). We compared the temporary temperature gradient gel electrophoreses (TTGE) to the routinely used polyacrylamid gel electrophoreses (PAGE). Five separate PCRs including five Vg-Primer (Vg-1-12) in combination with one GC-clamped Joining-Primer (Jg-1/2) were carried out and the products were separated with both TTGE and PAGE.
Results: With 4 primers each for the V and J section of the g-chain of the T-cell receptor in combination clonality could be proven in 76,5% of mycosis fungoides and 100 % of pleomorphic CTCL by using PAGE. The most common combination Vg-1-8 and Jg-1/2, however resulted only in 56,3% clonality in mycosis fungoides and 45,5% in pleomorphic cutaneous T-cell lymphoma. In the group of subacute dermatitis 3 clonal samples were found.
With PAGE in 4 cases of dermatitis clonality was found in contrast to 2 samples with TTGE. One tumor sample showed a polyclonal smear on PAGE but a clonal band on TTGE.
Conclusion: In order to successfully prove clonality it is essentiell to use primers for almost all possible rearrangements. TTGE is a well usable method and compared to other procedures cost efficient for analysis of T-cell clonality. Due to its high sensitivity PCR is a pillar in the diagnosis of CTCL. However, diagnosis merely based on clonality analysis is not possible as shown with "clonal dermatitis". In summary, diagnosis can only be defined by the clinical picture, histological result and clonality analysis.

P053

Scarring skin lesions of discoid lupus erythematosus are characterized by high numbers of granzyme B positive lymphocytes associated with strong expression of the type I interferon induced protein MxA

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Infiltrating T lymphocytes are considered to play a major pathological role in skin lesions of cutaneous lupus erythematosus (CLE), a cutaneous autoimmune disease of unknown etiology. Earlier histological studies revealed that the inflammatory infiltrate in CLE skin lesions is predominantly composed of T lymphocytes, with a slight majority of CD4+ over CD8+ T cells, but failed to explain the major difference between the two most common subsets of CLE, the skin atrophy characteristic for scarring chronic discoid LE (CDLE) and the non-scarring character of subacute cutaneous LE (SCLE). Because recent investigations highlighted the relevance of cytotoxic lymphocytes in autoimmune tissue destruction, we hypothesized that the scarring CDLE lesions might be caused by cytotoxic T lymphocytes.
We analyzed skin biopsies of alltogether 15 CLE patients (10 female, 5 male; lCDLE: n=5, dCDLE: n=5, SCLE n=5) and 5 control biopsies taken from healthy controls. The inflammatory infiltrate was characterized by immunohistochemistry. Monoclonal antibodies specific for CD3, CD4, CD8, CD20, CD68 and the cytotoxic molecule granzyme B (GrB7) were used for primary staining following the manufactures protocol. MxA (M143) labeling was performed on paraffin embedded tissue sections. Appropriate isotype matched controls were included. Secondary staining was performed using the LSAB2™ staining kit (DAKO™).
We found a strong expression of the cytotoxic molecule granzyme B in lesional lymphocytes of patients with lCDLE and dCDLE, which was significantly enhanced when compared to SCLE and healthy controls. The increased expression of granzyme B was closely associated with the lesional expression of the MxA protein, indicating lesional type I IFN production.
Our results provide evidence that scarring LE lesions are characterized by a high number of cytotoxic lymphocytes. We hypothesize that these cytotoxic T cells are responsible for the scarring character of skin lesions characteristic for CDLE.

P054

Enhanced type I interferon signaling and recruitment of chemokine receptor CXCR3 expressing lymphocytes into the skin following treatment with the TLR7-agonist imiquimod

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Imiquimod (Aldara™) is an immune response modifier approved for the topical treatment of external genital and perianal warts which can mediate regression of several cutaneous malignancies (basal cell carcinoma, Bowen#s disease, actinic keratosis, metastasis of malignant melanoma). Recently it was discovered, that imiquimod acts through the toll like receptor (TLR) 7. We hypothesize that TLR7-signaling strongly induces the production of interferon alpha (IFNα), which is able to enhance Th1-mediated cellular antiviral and antitumor immunity.
In the present study we analyzed the expression of MxA, a protein specifically induced by type I interferons, during topical imiquimod treatment in several patients suffering from different cutaneous malignancies (basal cell carcinoma, cutaneous metastasis of melanoma and breast cancer) and characterized the inflammatory infiltrate, along with the expression of chemokine receptor CXCR3, by immunohistochemistry.
Treatment with the TLR7 agonist imiquimod induced a significant lesional lymphocytic inflammation, associated with strong expression of MxA, indicating the induction of type I interferon signaling. The extent of lesional MxA staining closely correlated with the number of infiltrating T lymphocytes and the expression of the chemokine receptor CXCR3, characteristic for Th1-biased immune responses.
Our in vivo results suggest an important role for TLR7-induced production of type I IFN which links innate and adaptive immunity and promotes specific Th1-biased cellular immune response capable of eliminating cutaneous malignancies. MxA appears to be a valuable parameter to demonstrate IFN-type I expression in imiquimod therapy.

P055

TNF and IL-1-independent Hyperproliferation, Reduced Differentiation and Compromised Innate Immunity in Pseudomonas Aeruginosa Stimulated Epidermal Cells

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Pseudomonas aeruginosa (PA) delays wound healing. Bacterially induced inflammatory responses of the skin result in hyperproliferation and reduction of terminal differentiation. Stimulated TNF and IL-1 signalling pathways are known to mediate inflammatory processes. Our study examines whether PA compromises the TNF and IL-1/TLR family mediated infection defence in murine epidermal cells. Gene profiling was conducted using micro array technique from PAM212 keratinocyte cell line stimulated with PA supernatant. Customised micro arrays were designed for the use in dermatological research and contain oligonucleotide probe sets representing more than 400 transcripts. Six and 24 hours after PA supernatant treatment, we extracted total RNA and generated in vitro cRNA probes. Fragmented cRNA was hybridised and micro arrays were scanned and genes were filtered, signalling a consistent increase or decrease in three analyses. Selected gene expression was confirmed using RT-PCR and protein translation using FACS analysis. After 24 hrs of PA supernatant stimulation we found higher expression of proliferation associated mRNA of angiotensin-2 receptor and small proline-rich protein-1. TNF, MyD88, as marker of IL-1/TLR family signal transduction, and TLR9, known to recognise cg motives, showed no significant changes in gene or protein expression. Molecules of innate immunity lipopolysaccharide-binding-protein, mBD-2, -3, and -4 were down regulated by PA stimulation after 24 hrs. Down regulation of desmoglein-1, cathepsin D, and envoplakin represent reduced differentiation. PA induces proliferation in keratinocytes followed by a reduction of differentiation and compromised innate immunity. The hyperproliferation is independent of TNF and IL-1/TLR family signalling pathways. PA survives in wounds through secretion of molecules modulating inflammatory responses against bacteria.

P056

Differential expression of the lymphatic marker podoplanin in human squamous cell carcinomas and basal cell carcinomas

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The mucin-type glycoprotein podoplanin is specifically expressed by lymphatic, but not blood vascular endothelial cells, in culture and in tumor-associated lymphangiogenesis. Our previous studies showed that podoplanin deficiency results in congenital lymphedema and impaired lymphatic vascular patterning, and that podoplanin enhances migration and adhesion of endothelial cells by reorganization of the cytoskeleton. However, its expression in normal tissues and in human malignancies has remained unknown. Using tissue arrays of normal human tissues and of a number of human cancers, we found that - in addition to lymphatic endothelium - podoplanin is also expressed by bile duct cells of the liver, peritoneal mesothelial cells, osteocytes, glandular myoepithelial cells, ependyma cells, and by stromal reticular cells and follicular dendritic cells of lymphoid organs. In normal human skin podoplanin is focally expressed by basal keratinocytes, as shown using different antibodies. Immunohistochemical staining of paraffin embedded tissues revealed strongly induced podoplanin expression in 89% out of 87 squamous cell carcinomas (SCC). The expression pattern was dependent on the level of differentiation of the tumors. In contrast, in only one out of 74 basal cell carcinomas podoplanin expression was observed. Activation of the epidermal growth factor receptor (EGFR) has been proposed to enhance the metastatic potential of SCC, and podoplanin expression is known to be increased by EGF in keratinocytes in vitro. Therefore, based on the present findings of podoplanin expression in SCC we conclude that podoplanin may play an active role in epithelial tumor progression.

P057

No association between three xeroderma pigmentosum group C and one group G gene polymorphisms and risk of cutaneous melanoma

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Xeroderma pigmentosum (XP) patients exhibit a 1000-fold increased risk for developing skin cancers including malignant melanoma. We investigated the role of three variant alleles of the DNA repair gene XPC and one variant allele of the XPG gene in a hospital-based case-control study of 294 Caucasian patients from Germany with malignant melanoma and 375 healthy control individuals from the same area matched by sex. The polymorphisms G1580A (XPC exon 8; Arg492His), T1601C (XPC exon 8; Val499Ala), G2166A (XPC exon 10; Arg687Arg), and C3507G (XPG exon 15; Asp1104His) were not in linkage disequilibrium. The allele frequencies (cases : controls) were for 1580A 6.29% : 5.63%, for 1601C 79.08% : 78.28%, for 2166A 26.19% : 28.13%, and for 3507G 79.86% : 78.61%. We found no association of the homozygous 1580A, 1601C, 2166A, and 3507G genotypes with increased risks of melanoma: OR 1.254 (95%-CI: 0.486-3.217), OR 1.108 (95%-CI: 0.629-1.960), OR 0.817 (95%-CI: 0.490-1.358), and OR 1.168 (95%-CI: 0.670-2.044), respectively. Exploratory analyses of subgroups of melanoma patients compared to all controls indicated no association of these genotypes with increased risks for development of multiple primary melanomas (n=28), a negative family history for melanoma (n=277), melanomas in individuals with a low number of nevi (n=273), melanomas in individuals older than 55 years (n=142), and melanomas thicker than 1mm (n=126).

P058

Haplotype sharing analysis identifies a retroviral dUTPase as candidate susceptibility gene for psoriasis

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The PSORS1 mutation is assumed to reside within a region around HLA-C spanning 250 kb, termed RH (risk haplotype) 1/2. By re-analyzing a published data set with a previously developed method, the Haplotype Sharing Statistic (HSS), we confirm localization of PSORS1 to the RH1 region and refine its location to marker M6S168. We replicate this result in an independent patient sample. The target region harbors fragments of an HERV-K endogenous retrovirus. Two SNPs with alleles differening between high and low risk haplotypes are located within the HERV-K dUTPase. One of these encodes a predicted non-conserved Glu-Arg exchange. The HERV-K dUTPase is expressed in peripheral blood and in normal as well as lesional psoriatic skin. Our results indicate that an endogenous retroviral dUTPase constitutes a candidate gene for the PSORS1 mutation.

P059

Expression Profiling: A new Way in Skin Diagnostics?

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With the aim to develop new microarray based tools for the diagnosis and prognosis of skin diseases, we have applied two different expression profiling methods. Serial Analysis of Gene Expression (SAGE™) and PIQOR™ cDNA microarrays were used to generate the disease specific expression profiles of more than 600 individual skin biopsies affected with skin cancers or inflammatory skin diseases.

To identify disease specific expression profiles and potential marker genes, pools of patient biopsies were used to generate eight different SAGE™ libraries. Based on the results of the SAGE™ analysis, PIQOR™ cDNA microarrays were configured allowing the evaluation and extension of SAGE™ derived expression profiles based on single patient samples. This way, individual gene regulation could be distinguished from common expression changes.

Expression profiles are linked to extensive clinical data comprising patient histories, clinical parameters and in depth histological characterisations of each biopsy. An exhaustive analysis of all expression data, medical histories and histopathology data using bioinformatics should allow the stratification of patients and help to develop new diagnostic or prognostic tools.

The feasibility of this approach will be discussed based on the analysis of disease specific expression changes of selected gene groups.

P060

Molecular characterization of the first two cases of hepatoerythropoietic porphyria in Germany Germany - revision of two previous diagnoses

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Hepatoerythropoietic porphyria (HEP) arises from a severe decrease in the activity of uroporphyrinogen decarboxylase (URO-D), the fifth enzyme in the pathway of heme biosynthesis. This rare disorder is inherited in an autosomal recessive fashion and caused by recessive or compound heterozygous mutations in the URO-D gene. We report the cases of two patients who had previously been diagnosed clinically with congenital erythropoietic porphyria (CEP). CEP is also inherited in an autosomal recessive fashion but results from mutations in the uroporphyrinogen III synthase (URO-III-S) gene that encodes for the fourth enzyme in heme biosynthesis. Clinically, both CEP and HEP already manifest early in life with severe photosensitivity, exulcerations, mutilation, and scarring on the sun-exposed areas of the body and might therefore be easily confused. In both cases we were unable to detect disease causing mutation in the URO-III-S gene. This prompted us to study the URO-D gene for mutations in these cases since HEP seemed a good differential diagnosis in these families. Using PCR based techniques, we subsequently detected a novel missense mutation in exon 3 of the URO-D gene in both families. This example demonstrates that CEP and HEP might be confused regarding their clinical manifestation. In both cases, the advantages of molecular genetics allowed for revision of the initial diagnosis and was helpful in establishing the accurate diagnosis. To our knowledge, these are the first two families diagnosed with HEP in Germany. Further studies will show if the two families originate from a common ancestor.

P061

Transfection of human keratinocytes in vitro using non-viral vectors and FuGENE™ 6

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The skin, the most superficial part of the body, is a biologically very complex organ. Disruption of the normal function can led to several different diseases, both local skin diseases and systemic diseases, and these can be both inherited and acquired.
Therefore the skin is an attractive tissue site for development of new genetic therapeutic approaches for diseases that are amenable, in principle, to cutaneous gene transfer. Effective non-viral gene transfer systems have been developed that deliver genes to target cells without the inherent disadvantages of viral-based systems such as antigenicity and the potential for recombination with wild type viruses. Furthermore non-viral vectors do not have such safety concerns and are easy and safe to prepare.
In the present study we investigated transfection efficiency and toxicity of the transfection reagent FuGENE™ 6 in relation to keratinocytes. Human keratinocytes were isolated from foreskin, cultured and transfected with two reporter gene plasmids, coding for β-galactosidase (β-gal) and enhanced green fluorescent protein (EGFP). The production of β-gal protein was analysed by chemiluminescent assay and the number of cells producing EGFP was determined by flow cytometry. Our study demonstrates that FuGENE™ 6 is an efficient transfection reagent for non-viral gene transfer into human keratinocytes.

P062

Evaluation of cystatin M/E: a candidate for cornification disorders

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Cystatin M/E (CST6) is a novel cystein proteinase inhibitor showing a marked expression in the stratum granulosum of normal epidermis. Recently, it was demonstrated that autosomal recessive mutations in CST6 underlie the murine ichq phenotype, which is considered as a mouse model for human harlequin ichthyosis (HI). However, so far the study of human HI has neither revealed CST6 mutations nor striking alterations of cystatin M/E expression. In view of the mouse data and the epidermal expression pattern cystatin M/E is a strong candidate for cornification disorders especially for lamellar ichthyosis (LI), ichthyosis vulgaris (IV) and other ichthyoses.
We wondered whether there was an altered expression pattern of cystatin M/E in these diseases and whether mutations in CST6 were present in these keratinisation disorders. To address these questions we performed an immunohistochemical evaluation of a large set of cornification disorders (LI n=15; IV n=5; HI n=3; IFAP n=1) in comparison to healthy skin, atopic dermatitis and psoriasis vulgaris. For immunohistochemistry cryostat sections were stained with polyclonal rabbit anti-human cystatin M/E antibody. Presence of cystatin protein was visualized using fluorescence and light microscopy. Mutation analysis of the CST6 exons including exon/intron boundaries was performed in genomic DNA by direct sequencing of amplified DNA using Big Dye termination protocol.
Presence of cystatin M/E was observed in the stratum granulosum in most cases of LI including those showing transglutaminase-1 deficiency (n=4). Three patients with LI had a faint staining, while in HI protein expression was normal. In contrast, skin sections of IV showed a shift of the cystatin M/E expression to the stratum corneum. Interestingly one individual with LI presenting an enhanced epidermal expression of cystatin M/E showed a mutation on one allele in exon 1 (nt110-2A>2T) resulting in an amino acid change next to the reactive site. This mutation was absent in 90 different control chromosomes.
We conclude that cystatin M/E is associated with altered expression patterns in cornification disorders such as LI and IV and that these alterations are likely to play a role in the pathophysiology of these diseases. Whether the only mutation identified so far is causative for LI warrants further studies.

P063

Genotype/phenotype correlation of congenital ichthyosis caused by inactivating mutations of epidermal lipoxygenases.

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Autosomal recessive congenital ichthyosis (ARCI) forms a clinically and genetically heterogeneous group of severe hereditary keratinization disorders characterized by intense scaling of the whole integument, different in colour and shape and often associated with erythema. Up to the present, six loci for ARCI have been mapped. In order to characterize the mutational spectrum and function of epidermal lipoxygenases, we have analysed 143 cases of ARCI for mutations in ALOX12B and ALOXE3 on chromosome 17p13. Here we describe the molecular and clinical findings in 17 families with ARCI, originating from Central Europe, Turkey, and the Indian subcontinent, with mutations in ALOXE3 or ALOX12B. We have identified 12 novel point mutations in ALOX12B, one of which is a nonsense mutation, one is an intronic splice mutation, and all others are missense mutations, and six different mutations in ALOXE3, of which one is a potential exonic splice mutation. To verify the destruction of splice sites we have established an in-vitro splice assay. The gene products of ALOXE3 and ALOX12B, epidermis-type lipoxygenases 12R-LOX and eLOX-3, respectively, are preferentially synthesized in the skin and subsequent members of the same pathway converting arachidonic acid via 12(R)-HPETE to the corresponding epoxyalcohol, 8(R)-hydroxy-11(R),12(R)-epoxyeicosatrienoic acid. In order to assess the impairment of enzyme activity using the genuine substrates, we have expressed the mutated genes in vitro and developed a lipoxygenase activity assay. The analysis of reaction products demonstrated that all but one recombinant mutants were enzymatically inactive. Characterization of disease causing mutations in ALOXE3 and ALOX12B and the resulting ARCI phenotypes in the families described did not result in clear diagnostic criteria, however, we have revealed a first correlation between genetic findings and the clinical presentation of ichthyosis.

P064

An internet-based database on the analysis of autosomal recessive congenital ichthyoses and other inherited disorders of keratinization

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Autosomal recessive congenital ichthyosis (ARCI) is a clinically and genetically heterogeneous disorder characterized by generalized scaling of the skin and erythema. Color and shape of the scales and extent of erythema are highly variable, as well as a number of further features. A consistent genotype/phenotype correlation was not identified so far. In order to store and provide data from patients with ARCI and other related skin disorders, we have now developed a concise database service. Different phenotype variables, family history, pedigree, biochemical and histopathological data are stored for each sample. The family history will be automatically transferred to linkage compatible data formats and graphical output of the pedigree, clinical pictures will be included and presented as thumbnails. The service runs on an Apache 2 web server and is powered by a MySQL database management system and accessible through the web using an HTTP interface. For data protection reasons, sample names are stored on a separate server connected with the database by anonymous 32bit sample IDs. Data requests are handled semi-automatically via email using temporary IDs, each valid for immediate access and one dataset. The database stores genotyping data for indirect analysis of all known ARCI loci as well as mutation data obtained by direct sequencing. Further data are being collected within the German "Network for Ichthyoses and Related Keratinization Disorders", which aims at comprehensively recording and characterizing such families. The service is open to external users on a collaborative basis and includes different user levels. The main purpose of the database is to assist the work of research groups by providing a common platform for data collection and exchange. Since ARCI and other keratinization disorders are rare, the database not only provides a mutation survey but is valuable for systematically collecting data. Thus, it helps to generate criteria for analyzing a potential genotype/phenotype correlation and further characterization of the phenotypic outcome of specific mutations.

P065

Characterization of retroviral particles derived from human melanoma cells

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The human genome harbors 1-2% of endogenous retroviral sequences. Human endogenous retroviruses (HERV) represent a cellular reservoir of potentially pathogenic retroviral genes. Recently we have shown that human melanoma cells produce retrovirus-like particles that exhibit reverse-transcriptase activity and package sequences homologous to human endogenous retrovirus K (HERV-K). In this study we analyzed the diversity of particles derived from supernatants of different melanoma cell lines. Viral particles were characterized on iodixanol step gradients. Gradient fractions were collected, their density was determined and RT activity of each fraction was analysed by PERT. Viral RNA of each fraction was isolated and amplified by RT-PCR with pol-specific primers. Amplification products were sequenced and compared to the corresponding sequence of HERV-K. For further sequence analysis of the melanoma-associated retrovirus, genomic DNA and cDNA derived from MDBK (Madin-Darby bovine kidney) cells exposed to human melanoma derived particles were amplified by PCR with HERV-K specific primers. Four amplification products covering the whole proviral coding sequence were obtained and sequenced. Conserved domains for a matrix protein, nucleocapsid protein, dUTPase, protease, reverse transcriptase, RNase H, integrase and of gp36 envelope protein were found. This result shows that all proteins necessary to form a functional virus are encoded.

P066

HHV8-positive Kaposi sarcoma in a long time HIV-exposed but -uninfected patient carrying the CCR5-promoter mutation A59029G

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The 49 year old male caucasian patient presented with several slowly growing dark-reddish nodules on his lower left leg which were increasing in size for the last 18 months but were otherwise asymptomatic. Two distinct biopsies showed Kaposi#s sarcoma (KS). His personal history revealed homosexual practices without protection with high-risk for HIV transmission for more than 20 years. Repeated HIV 1/2-testing including routine ELISA, Western Blotting, and PCR-analysis, however, had shown negative results and were negative at the time of presentation. His peripheral lymphocytic repertoire also showed no abnormalities with a peripheral CD4 count of 572 / mm3. Serological screening for HHV8 revealed a low anti-HHV8 IgG titer in the serum. PCR-analysis for HHV8 was found positive within the KS-lesions but was found negative in PBMCs. At this point a localized cutaneous HHV8-positive Kaposi sarcoma in a patient with high risk HIV-exposure but without detectable HIV infection was diagnosed. We started a low-energy X-ray therapy followed by a interferon α 2b treatment. We next tried to identify a reason for his protection to HIV transmission despite his prolonged high risk exposure and despite the obvious transmission of HHV8. Several genetic dispositions have been described in long time HIV-protected individuals including the Δ32-mutation of the CCR5 co-receptor. We thus tested PBMCs from our patient for the expression of CCR5 by FACS analysis. Interestingly, the expression of CCR5 on peripheral PBMCs was very low as compared to control PBMCs. A screening for known mutations of the CCR5-gene family showed negative results for the Δ32-mutation of the CCR5 co-receptor and the CCR2-variant G46294A but yielded the homozygous variant A59029G of the CCR5-promoter region.
This is to our knowledge the first report of a protective effect against HIV-transmission by the A59029G CCR5-promoter-variant.

P067

Xeroderma pigmentosum C gene polymorphisms and alternative splicing in patients with multiple sporadic primary cutaneous melanomas

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In the rare autosomal recessive disease xeroderma pigmentosum (XP) defects in nucleotid excision repair genes lead to an >1000-fold increased risk for developing skin cancer including melanoma. Recently three polymorphisms in the XPC gene (intron 11 C-6A, exon 15 A2920C and intron 9 poly AT) which are in linkage desequilibrium have been described. The intron 11 C-6A polymorphism within the XPC intron 11 splice acceptor site has been described to lead to an increased exon 12 skipping in the XPC mRNA which results in diminished DNA repair function. To investigate if this may contribute to the development of cutaneous melanoma we assessed these three XPC gene polymorphisms and expression of the alternatively spliced XPC mRNA variant in a high risk group of 27 patients with at least two cutaneous melanomas without familial background and 30 healthy age-, gender and ethnicity-matched individuals.
In all 57 individuals these three XPC polymorphisms were in linkage desequilibrium consistent with the literature. Using quantitative real-time PCR we could confirm the finding that the XPC intron 11 C-6A polymorphism leads to an increased expression of the exon 12 deleted XPC mRNA isoform in an allele dosage dependent manner. Overall, the relative expression of the XPC mRNA isoform was 1,9-fold higher in individuals carrying the XPC intron 11 C-6A polymorphism in both alleles (homozygous A/A genotype) and 1,6-fold higher in heterozygous C/A genotype carriers compared to individuals with the homozygous C/C genotype in this group.
Referring to a possible association with melanoma we detected the homozygous A/A genotype in 9 of 27 (33,33 %) melanoma patients but only in 4 of 30 (15,38 %) healthy controls. Consistent with this finding the XPC mRNA isoform was expressed in a relative amount of 1,22 % of wildtype XPC mRNA in melanoma cases compared to only 1,16 % in controls. Although statistically not significant at this stage these findings show an interesting tendency which has to be confirmed by a larger cohort. This might result in the hypothesis that the presence of the XPC intron 11 C-6A polymorphism which leads to an increased expression of an exon 12 deleted XPC mRNA isoform may contribute to the pathogenesis of cutaneous melanoma and may represent a biomarker for melanoma susceptibility.

P068

Activated platelets are important mediators of cutaneous inflammation

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Injury of the vessel wall leads to activation of coagulation, which triggers immune responses. Since injury may bear the risk of infection, this interaction may be beneficial. However, a pro-coagulant state may also have an implication in disease. Using intravital microscopy, we have shown, that activated, not resting, platelets form platelet-leukocyte aggregates in the blood stream. Aggregate formation was P-selectin-dependent. As a consequence, leukocyte rolling in the murine skin microvasculature was increased. Rolling does not per se lead to greater extravasation, since extravasation depends on subsequent activation, firm adhesion and transmigration. We therefore investigated the role of platelets in a murine model of contact hypersensitivity (CHS) using DNFB. Induction of thrombocytopenia before challenge lead to a reduced CHS response, indicating, that platelets are important in mediating cutaneous inflammation (Δ ear swelling: 44.5$\pm$41.5cm x10-3 vs. 23.8$\pm$29.1 cm x10-3). To analyse the impact of platelet P-selectin-mediated leukocyte rolling on development of a CHS response we treated thrombocytopenic mice with serotonin depleted, resting or activated platelets immediately before challenge with DNFB. To further minimize the effect of serotonin, mice additionally received the serotonin-antagonist ketanserin at 3.2mg/kg body weight. In comparison to control animals, ear swelling response in mice receiving resting platelets was similar to thrombocytopenic mice (23.6$\pm$17.0cm x10-3). In contrast, ear swelling responses were restored in mice receiving activated platelets (48.6$\pm$13.6 cm x10-3). To clarify the role of platelet P-selectin in cutaneous inflammation we are generating mice deficient for platelet P-selectin while still expressing endothelial P-selectin using bone marrow transplantation. In summary, platelet P-selectin expression contributes to leukocyte extravasation and platelets must be considered important effector cells of the (skin) immune system.

P069

Junctional Adhesion Molecules (JAM)-B and -C contribute to leukocyte extravasation in the skin and mediate cutaneous inflammation

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Leukocyte extravasation is a highly regulated process, in which transmigration is the final step. Transmigration depends on molecules located at borders of endothelial cells; e.g. Junctional Adhesion Molecules (JAM-A, -B and -C). In vivo blockade of JAM-A leads to decreased migration of monocytes. In contrast, the role of JAM-B and -C in development of cutaneous inflammation is unknown. We therefore elicited an allergic contact dermatitis (CHS) in mice using DNFB. Immunofluorescent staining revealed a constitutive JAM-B and -C expression in normal mouse skin. Functional studies, in which either JAM-B or -C neutralizing antibodies were injected in sensitised mice prior to allergen challenge showed a dose dependent reduction of the contact dermatitis. Measured endpoints were ear swelling response and infiltration determined in H&E stained sections and as elastase/peroxidase activity in homogenized samples. Combined antibody treatment leads to synergistic inhibition of allergic contact dermatitis, indicating that JAM-B and -C have overlapping, but distinct functions. In addition to their role in transmigration JAM-B and -C might also contribute to leukocyte rolling. E.g. JAM-B binds VLA-4 which mediates rolling and firm adhesion in the skin microvasculature via interaction with VCAM-1. We therefore investigated the impact of JAM-B blockade on leukocyte rolling in murine skin using intravital microscopy. Rolling of endogenous leukocytes was significantly reduced from 23.8±7.3% to 13.4±10.9% after infusion of a function blocking JAM-B antibody. In conclusion, interactions with JAM-B and -C are essential for development of cutaneous inflammation - and JAM-B and -C may mediate further steps in leukocyte recirculation.

P070

A gene signature of inhibitory MHC receptors identifies a novel tolerogenic subset of regulatory DC

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Dendritic cells (DC) are crucial gatekeepers in regulating immunity. Whereas immunostimulatory myeloid DC (DCims) potently promote proinflammatory immune responses, IL10-modulated regulatory myeloid DC (DCreg-IL10) mediate tolerance induction. In order to elucidate the mechanisms by which DCreg-IL10 induce T cell tolerance, comparative gene expression profiling was done using Affymetrix U133 microarrays. In DCreg-IL10 (DCims) 256 (93) genes were up-regulated. Among the immune response genes over-expressed in DCreg-IL10, 9 ITIM-containing inhibitory molecules (ILT2, ILT3, ILT4, ILT5, LILRB5, DCIR, PILRA, FcgRIIB, SLAM) were found. Phenotypic analysis revealed that DCreg-IL10 comprise an ILT2high CD83low subset which was shown to be the most effective in inducing persistent T-cell anergy after restimulation with DCims as in-vitro measure of tolerance induction. Thus, ILThigh DCreg-IL10 have a potential for future therapeutic applications targeting alloand autoreactivity.

P071

Inhibition of NADPH-oxidase abolishes leukocyte-dependent dermal-epidermal separation induced by autoantibodies from patients with bullous pemphigoid

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Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease associated with tissue-bound and circulating autoantibodies to the dermal-epidermal junction. Patients# autoantibodies are directed to BP180/type XVII collagen and induce dermal-epidermal separation in cryosections of human skin when co-incubated with human leukocytes. In this experimental model of BP, recruitment and activation of granulocytes by IgG-containing immune complexes with subsequent release of leukocyte proteases are prerequisites for subepidermal split formation. In addition to proteases, leukocyte-derived reactive oxygen species (ROS) represent key effectors of tissue injury in diseases associated with infiltrating granulocytes. In the present study, we investigated the role of ROS for blister formation induced by BP autoantibodies. Leukocytes were recruited to the dermal-epidermal junction in cryosections incubated with IgG purified from BP patients, but not from healthy donors, and induced the in situ reduction of nitro blue tetrazolium to formazan precipitates suggesting the release of ROS from leukocytes. In order to assess more precisely the pathogenic significance of ROS produced by leukocytes stimulated by IgG from BP patients, we blocked the activity of nicotinamide-adenine-dinucleotide-phosphate (NADPH)-oxidase using diphenylene iodonium (DPI). Interestingly, BP autoantibodies failed to induce subepidermal splits in cryosections incubated with leukocytes in the presence of DPI. The capacity of viable leukocytes to produce ROS was abolished by DPI as demonstrated by both the superoxide dismutase-inhibitable reduction of cytochrome c and the absence of formazan precipitates in cryosections. In conclusion, inhibition of ROS generation by leukocytes abolishes blister formation induced by BP autoantibodies. These new insights into the pathomechanisms of blister formation in BP may contribute to the development of more specific therapeutic tools for this disease.

P072

Intravenous immunoglobulins block tumor necrosis factor-α and interleukin-12 production by proinflammatory human blood dendritic cells

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Intravenous immunoglobulins (IVIG) are effective in the treatment of autoimmune diseases and systemic inflammatory disorders, but questions remain concerning the mode of action. When immunoglobulins are administered intravenously they first make contact with leukocytes in the blood. Among the blood leukocytes, M-DC8+ dendritic cells stand out by their strong capacity to produce tumor necrosis factor-α (TNF-α). In addition, M-DC8+ DC produce ten times higher interleukin-12 (IL-12) levels than monocytes or CD11c+/M-DC8- blood DC, they are potent inducers of Th1 cells and therefore may be relevant for the pathogenesis and progression of autoimmune diseases.
Here we demonstrate the functional inhibition of M-DC8+ DC by therapeutic doses of IVIG. Native M-DC8+ blood DC were isolated by magnetic cell sorting from buffy coats of healthy donors to a purity of >95% and treated with different doses of IVIG for a period of 6 hours. After washing the cells to remove IVIG, cytokine secretion of DC was induced by stimulation of CD40, TLR-2, TLR-3 and TLR-4 with CD40L-transfected fibroblasts, PGN, Poli I:C and lipopolysaccharide (LPS), respectively. With all stimuli used, the pretreatment of the M-DC8+ DC with IVIG led to a strong and dose-dependent reduction of IL-12p70 and TNF-α production. 80% reduction of IL-12 and TNF-α secretion was observed after maximal stimulation with LPS (TLR-4 ligand) combined with interferon-γ. Unstimulated M-DC8+ DC undergo in vitro a "spontaneous" maturation with marked phenotypic changes already seen after 2 hours. In the presence of IVIG, this rapid upregulation of the maturation marker CD83 and of the costimulatory molecule CD86 as well as the shedding of the FcγRIII (CD16) was reduced. Furthermore, IVIG impaired the adherence capacity of DC depending on the time elapsing (57% reduction after 30 min., 33% after 2 hours). The suppressive effect of IVIG on M-DC8+ DC could not be attributed to IL-10 which was not significantly induced, also an upregulation of the inhibitory molecules ILT-2 and ILT-4 was not seen. In conclusion, we here describe the profound inhibitory effect of IVIG on the highly proinflammatory population of M-DC8+ DC and hereby point out an important target for the anti-inflammatory action of IVIG in human blood.

P073

Osteopontin Function for DC Migration and Activation is Modulated by Thrombin Cleavage

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The phosphoglycoprotein Osteopontin (OPN) is a chemotaxin for Langerhans-/Dendritic Cells (DC) that crucially affects their migration to lymph nodes during the sensitization phase of allergic contact hypersensitivity. OPN has a modular structure with multiple functional domains. Its biological activity is influenced by its state of glycosylation, phosphorylation and proteolytic fragmentation. Thrombin cleavage of OPN, results in two fragments of similar size but with distinct biological functions. The N-terminal fragment contains an RGD fÑv-integrin binding sequence, while the C-terminal fragment contains the CD44 binding domain. Here we investigated the secondary structure of eukaryotic OPN and compared its biological activity with thrombin cleaved fragments and a prokaryotic OPN-GST-protein in its capacity to induce DC activation and migration. 6xHistidin(His6)-tagged recombinant full length mOPN was expressed by HEK-293-EBNA cells and was purified by its His6-tag. This mOPN was cleaved by thrombin and fragments were separated by N-terminal His6-tag. Analyzing the circular dichroism of each protein, we found all variants to be unstructured in solution showing a spectrum characteristic for a random coil. Investigating the function of the eukaryotic and prokaryotic OPN, we found both forms to induce DC activation, as measured by their expression of MHC-II and CD86. Interestingly, the DC activating effect of eukaryotic OPN was more pronounced. In Chemotaxis assays with BM-DC comparing the promigratory capacity of full length OPN and the generated fragments, the eukaryotic full length OPN was the more potent chemotaxin. The most important domain mediating DC-migration seems to be located within the C-terminal half of the molecule which contains the CD44 binding domain, because the integrin binding N-terminal fragment was less chemoattractive. In conclusion we found eukaryotic OPN to have a random coil structure in solution. Functionally, OPN cleavage, in vivo occurring at sites of inflammation may influence the outcome of an immune response by differentially modulating DC activation and migration.

P074

Induction of regulatory/suppressor T cells by IL-10-treated dendritic cells and TGF-ß

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In grass pollen allergic individuals T cell anergy can be induced by IL-10-pretreated dendritic cells (DC) resulting in a decreased proliferation and Th1 as well as Th2 cytokine production. This study was set out to further analyze whether such anergic T cells are able to suppress peripheral T cells and to analyze the role of TGF-ß as potential inducer of regulatory T cells in this system. Freshly isolated CD4+ T cells from grass pollen allergic donors were stimulated with autologous mature monocyte-derived allergen-pulsed dendritic cells in the presence or absence of CD4+ T cells of the same donor previously cultured with IL-10-treated DC with or without addition of TGF-ß. Anergic T cells induced by IL-10-treated DC enhanced IL-10 production and strongly inhibited IFN-gamma production of peripheral CD4+ T cells while proliferation and Th2 cytokine production were only slightly reduced. The addition of TGF-ß had an additional effect leading to a significant suppression of Th2 cytokine production. These data demonstrate that regulatory/suppressor T cells that also suppress Th2 cytokine production are induced much more efficiently by DC that have been pretreated with IL-10 and TGF-ß. This might be exploited for future strategies aiming at the downregulation of allergic immune responses in atopic diseases.

P075

β2-integrins but not ICAM-1 are mandatory in LcV for PMN recruitment and for support of FcγR-dependent functions

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β2-integrins are mandatory for recruitment of granulocytes and share ICAM-1 as a ligand. They are also involved in functions such as phagocytosis and generation of oxygen radicals.
The Arthus reaction is an experimental model for LcV due to vascular deposition of IC. It is dependent on recruitment and degranulation of PMN. We wondered i) whether PMN damage endothelial cells really from the luminal side where their toxic contents could easilly be spilled away by blood flow, or rather from the abluminal side, and ii) to which extent ICAM-1 and β2-integrins contribute to full recruitment of PMN, degranulation and oxidative burst.
We addressed these questions by using mice deficient of or hypomorphic for the common βchain of the β2-integrins, as well as mice deficient of ICAM-1.
The Arthus reaction was elicited and quantitated by injection of FITC-BSA iv and anti-BSA sc into ears. In CD18-/- mice haemorrhage and tissue damage were almost absent, corresponding to the absence of early PMN infiltration. In contrast the typical clinical and histological picture of LcV was observed not only in WT mice, but also in CD18 hypomorphic and ICAM-1-deficient mice. Thus, while CD18 is mandatory for LcV, expression of only 1-10% of the protein is sufficient to mediate a full reaction. Deficiency of its primary ligand, ICAM-1, does not impair LcV, indicating full compensation by other ligands.
Functionally, we found that the absence of β2-integrins reduces, but does not prevent degranulation of PMN in response to bound IC (elastase assay). Thus, the lack of LcV in CD18-/- mice is not due to incapability to degranulate. This was further confirmed by the unimpaired LcV in CD18 hypomorphic mice.
LcV could be restored in CD18-/- mice by injecting CD18+/+ viable PMN in CD18-/- mice iv, but not by injecting them sc, thus placing them at the luminal side while bypassing diapedesis.
We conclude that i)although IC deposition occurs at both sides of the vessel wall, the damaging processes occur only at the luminal side, apparently when transmigrating PMN bind and react to IC trapped between endothelial cells and basal lamina; ii) β2-integrins support, but are not mandatory for signalling of FcγR; iii) ICAM-1 is not mandatory for recruitment of PMN and can be fully compensated in the Arthus reaction.

P076

GNB3 C825T polymorphism and response to anti-retroviral combination therapy in HIV-1-infected patients

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Abstract
Objective: Treatment success of HIV 1 infected patients has been shown to be influenced by genetic host factors. Investigations suggest that certain aspects of the strength of the human cellular immune response at least in part depend on the efficacy of G protein activation associated with C825T genotype status We investigated the association of GNB3 C825T genotypes with treatment response to highly active anti-retroviral therapy (HAART) in HIV-1-infected patients.
Methods: Analyses were based on a cohort of 55 HIV infected patients whose treatment was changed due to therapy failure. Viral load, CD4+ and CD8+ cell counts were determined before therapy and after 8 weeks and 16-32 weeks, respectively. GNB3 genotyping was performed using pyrosequencing. Chemotaxis of purified CD4+ cells was quantified in a Boyden chamber using stromal cell-derived factor 1á (SDF-1á) as a stimulus.
Results: Age gender, route of infection, treatment, and baseline values for viral load and CD4+ and CD8+ cell counts were not associated with GNB3 genotypes (10 TT, 24 TC, 21CC). After 8 weeks of treatment viral load was lowest in TT genotypes (log copies/ml: TT: 1.88 ± 0.32; TC: 2.49 ± 0.24; CC: 3.06 ± 0.22; p = 0.039) and this trend tended to persist until final visit. Unexpectedly, TT genotypes had lowest CD4+ cell counts at final visit (TT: 165.3 ± 34.0; TC: 414.4 ± 72.1; CC: 441.5 ±70.9; p = 0.047). SDF-1á -stimulated chemotaxis was reduced in CD4+ cells from HIV-1-infected patients compared to healthy controls. Still cells from TT genotypes displayed strongest chemotaxis.
Conclusions: The GNB3 C825T polymorphism appears associated with short-term success of HAART treatment in HIV-1-infected patients. Despite strongest suppression of viral load CD4+ cells do not increase in patients with TT genotype.

P077

Strong, persistent Melan-A/MART-1-peptide-specific immune responses after dendritic cell vaccination of stage II melanoma patients

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Dendritic cells have been shown to elicit expansion of tumor-specific CD8+ T cells in advanced (stage IV) melanoma patients. However, apart from occasional regressions of metastases in most patients these T cell responses were transient and non-protective, suggesting immune escape or tolerance induction by the tumor. Stage II melanoma patients remain at risk of tumor recurrence after primary tumor excision. Nevertheless, these patients are expected to be fully competent to mount T cell responses against tumor-associated antigens and therefore may derive survival benefit from therapeutic vaccination.
We used mature, monocyte-derived DC to generate CD8+ T cell responses against a Melan-A/MART-1-peptide in 14 stage II melanoma patients after resection of the tumor. Vaccination induced strong tumor-peptide-specific DTH reactivity that was impressively long-lived and could be detected up to twelve months after the last immunization. Further, vaccination resulted in a significant expansion of Melan-A/MART-1-specific CD8+ T lymphocytes. Along their expansion in the majority of patients, these cells acquired the capacity to secrete IFN-gamma and demonstrate improved proliferation in response to peptide stimulation in vitro, proving differentiation into effector/memory cells.
This study demonstrates for the first time that vaccination with peptide-loaded dendritic cells can induce strong and persistent tumor-specific CD8+ T cell responses in stage II melanoma patients suggesting that protective T cell immunity can be achieved.

P078

Dendritic cells govern induction and reprogramming of tissue-selective T cell trafficking by soluble factors

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Effector/memory T cells show tissue-selective trafficking by expression of distinct arrays of homing receptors like E-selectin ligands (E-lig) and chemokine receptors like CCR4 for skin- and integrin $\alpha4\beta7$ and CCR9 for small intestine-homing. We and others have established that these homing subsets are rapidly generated during first antigen contact under the influence of tissue-specific DC and the local tissue microenvironment (LTM). However, the crucial factors for the generation and the stability of polarized homing subsets are not known, yet. For therapeutical reasons it will be of central interest, if this tissue tropism of T cells is terminally imprinted or can be switched by alternative tissue-specific signals in a reprogramming process.
Using conditioned media from tissue-specific DC/T-cell cultures we demonstrate here, that soluble inductive as well as suppressive factors released by the DC efficiently regulated homing receptors on CD8+ T cells primed with anti-CD3 antibody. Using IL-12 p35/p40 KO mice we found that IL-12, unlike suggested by others, is not a crucial factor for the induction of functional E-lig on T cells by Langerhans cells (LC) in vitro and in vivo.
Interestingly, when tissue-specific DC were injected via different routes, they could not promote their corresponding homing receptors in a foreign tissue, demonstrating a functional dominance of the LTM. Furthermore, we generated E-lig.high (skin-homing) or $\alpha4\beta7$/CCR9high (gut-homing) T cells in vitro and in vivo and restimulated them with the `opposite tissue#-derived DC. Importantly, LC and skin-associated DC downregulated $\alpha4\beta7$ and induced high levels of E-lig whereas gut-associated DC changed homing receptor expression on T cells to $\alpha4\beta7$high/E-lig.low.
These results suggest that polarized T cell homing patterns are not terminally imprinted but can be switched by DC. Managing flexible tissue-targeting of T cells may provide new tools for immunotherapy of allergies, cancer and autoimmunity.

P079

The anti-psoriatic efficacy of the fully human anti-ICAM-1 antibody MOR102 may depend on blocking the interaction between lymphocytes and keratinocytes

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Skin-homing memory T lymphocytes play an important role in the pathogenesis of psoriasis by interacting with vascular adhesion molecules and trafficking into lesional skin. Targeting intercellular adhesion molecule 1 (ICAM-1) is an attractive option since this molecule is critically involved in leukocyte adhesion and extravasation as well as in lymphocyte activation.
We have selected the fully human monoclonal antibody MOR102 against ICAM-1 from the Human Combinatorial Antibody Library (HuCALÒ) and then converted to the human IgG4 format. The antibody demonstrated efficient inhibition of lymphocyte adhesion to recombinant ICAM-1 in-vitro and reduced lymphocyte proliferation in mixed lymphocyte cultures by ~50 %. FACS analyses demonstrated its ability to bind ICAM-1 on stimulated HaCaT keratinocytes. Subsequently, the in vivo efficacy of MOR102 was tested in the psoriasis SCID mouse model. Intraperitoneal injection of 10 mg/kg of MOR102 antibody every other day over a period of 4 weeks resulted in reconstitution of orthokeratotic differentiation and a significant ($p<0.05$) reduction in epidermal thickness as well as marked reduction in the inflammatory infiltrate. Immunohistochemistry showed in-vivo binding of MOR102 to ICAM-1 expressed on lesional keratinocytes, thus potentially preventing efficient co-stimulation of local T cells.
Based on the efficacy of the fully human monoclonal antibody MOR102 demonstrated in vitro as well as in vivo in the psoriasis SCID mouse model, initiation of clinical studies is feasible.

P080

MACROPHAGES PLAY A CENTRAL ROLE IN THE PSORIASIFORM SKIN DISEASE OF CD18 HYPOMORPHIC PL/J MICE

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In the CD18 hypomorphic PL/J mouse model, a mutation in CD18 gene leads to reduced expression of the CD18 protein to 2-16% of wild type level. PL/J mice with this mutation develop a dermatitis resembling human psoriasis. Immunostaining revealed a significant increase of macrophages in lesional skin as well as skin draining lymph nodes of CD18 hypomorphic compared to wild type mice. In vivo depletion of macrophages by clodronate resulted in a significant reduction of the psoriasiform skin disease in seven mice, whereas PBS-treated CD18 hypomorphic mice did not show any improvement of the psoriasiform phenotype. These data suggest that macrophages are centrally required for the development and maintenance of the psoriasiform skin disease in CD18 hypomorphic PL/J mice.
Monocyte chemoattractant protein 1 (MCP-1) has been indicated to be essential for monocyte recruitment in several inflammatory models in vivo. In lesional skin of psoriasis patients, MCP-1 has also been reported to be significantly enhanced. Compared to wild type mice, in psoriasiform skin of CD18 hypomorphic mice both MCP-1 mRNA and protein level, are increased. To investigate if MCP-1 is sufficient to induce the psoriasiform dermatitis, we subcutaneously injected rJE/MCP-1 into healthy CD18 hypomorphic mice. However, after six weeks of observation MCP-1 dependent monocytes failed to trigger the psoriasiform skin disease, suggesting that apart from MCP-1 other factors are required for the precipitation of the psoriasiform phenotype.
Macrophages express MHCII in skin draining lymph nodes of CD18 hypomorphic mice. To address if these macrophages are antigen presenting cells in inflamed skin draining lymph nodes, inflamed lymph nodes sheets were stained by MOMA-2 and anti-CD3 MAbs. As macrophages are found to be located mainly in the non-T cell area, it is unlikely that these cells act as initial antigen presenting cells. Furthermore the F4/80 and MHCII double positive cells are increased 100 times in inflamed lymph nodes of CD18 hypomorphic mice compared to wild type mice. 80% of them could not be depleted by clodronate. Hence, these cells are likely to be Langerhans# cells acting as antigen presenting cells. Accordingly, these data suggest that macrophages rather play an important role in maintaining the disease than inducing it via antigen presentation.

P081

Absence of autoantibodies against recombinant fibrillin-1 polypeptides in systemic scleroderma patients

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Autoantibodies to fibrillin-1 have been described in the tight-skin mouse, systemic scleroderma, mixed connective tissue disease, and primary pulmonary hypertension syndrome. In patients with scleroderma, the frequency of positive antibodies against a short recombinant 30 kd long fibrillin-1 polypeptide was reported to reach 31% in Caucasians and 87 % in Japanese. Until now it is unclear whether this immune response plays a primary role in the disease pathogenesis or is a secondary phenomenon. In the present study, we analyzed the frequency of antibodies to overlapping recombinant fibrillin-1 polypeptides in German patients with systemic scleroderma.
Screening of fibrillin-1 antibodies was performed in 40 sera from scleroderma patients and in 40 healthy controls. Overlapping recombinant human fibrillin-1 polypeptides covering the N-terminal half (rFBN1-N) and the C-terminal half (rFBN1-C) of human fibrillin-1 were produced in human embryonic kidney cells 293. Microtiter plates were coated with the recombinant polypeptides and incubated with 1:100 diluted sera. Positive binding was declared as ≥ 2 SD above the mean of the control group.
Correct three dimensional structures of recombinant fibrillin peptides was shown by ligand binding assays, electron microscopy and western blotting. Surprisingly, none of the sera of patients with systemic sclerosis showed antibodies neither to the N-terminal nor the C-terminal recombinant fibrillin-1 polypeptide. Furthermore, in sera from tight skin mice no antibodies against recombinant fibrillin-1 protein could be detected.
These data demonstrate the absence of autoantibodies against recombinant fibrillin-1 polypeptides in systemic scleroderma patients of a european caucasian background and in tight skin mice. Since the correct three dimensional folding of the recombinant protein used in this study was substantiated by several independent methods, we hypothesize that the occurrence of autoantibodies against fibrillin in systemic scleroderma is a secondary process and does not play a primary role in the pathogenesis of the disease.

P082

Skin-homing T cells have characteristics of side-population cells

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The initial purpose of this study was to investigate the immunosenescence skin homing T-cells expressing the cutaneous lymphocyte antigen (CLA). Peripheral blood lymphocytes from 72 healthy individuals (33 male and 39 female, median age: 54 years, range: 18-94 years) were investigated. The expression of CD28, CD45RA, CD45RO as well as intracellular interferon-γ (IFNγ) and interleukin-4 (IL-4) formation of CLA+ "skin homing" T-cells was analyzed. Additionally, T cells were detected immunohistologically in skin specimens from 15 young and 15 old healthy individuals. Relative telomere length (RTL) was measured by fluorescence in situ hybridization using flow cytometry (flow FISH). The total number of CLA+ T-cells remains constant with increasing age. In contrast to peripheral blood T-cells (CD3+, CLA-), which showed significantly decreased CD28 and CD45RA expression in donors >60 years, no age related alterations of either CD28+ CLA+ T-cells or CD45RA+ CLA+ T cells were observed. In the group of donors >60 years, the proportion of intracellular IFNγ producing CD3+ CLA- cells significantly increased whereas the number of IFNγ and IL-4 producing CLA+ T cells was not affected by age. After stimulation with phytohemaglutinine (PHA) or staphylococcal enterotoxin B (SEB), CLA+ T cells from old individuals did not show reduced response compared with CLA+ T cells from young donors. Additionally, the counts of T cells in healthy skin from young and old adults were statistically not different. Furthermore, RTL was significantly shortened in enriched CD45RO+ CLA- T cells from healthy old individuals but not in aged CLA+ T cells. Furthermore, analysis by flowcytometry demonstrated that CLA+ T cells belong to the population of central memory T cells (CD45RO+ CCR7+) and have characteristics of lineage-negative side-population cells, which are defined by their active efflux of Hoechst 33342 dye via a p-glycoprotein multidrug/ATP-binding cassette transporter protein (ABCG2). The present data could explain why CLA+ T cells might be a T cell subpopulation which does not undergo immunosenescence.

P083

Oligodeoxynucleotides containing CpG dinucleotides mediate an anti-inflammatory response in epithelial cells

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Unmethylated CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides (ODN) are described to activate immune cells such as dendritic cells and macrophages, via Toll-like receptor 9 (TLR-9). It is known that CpG-DNA enters immune cells via endocytosis and reaches endosomal compartments where it binds to the Toll-like receptor 9. This activation leads to a Th-1-cytokine pattern in T-helper cells.
To test whether this concept suits also to epithelial cells, we incubated normal human keratinocytes, HaCaT cells or human embryonic kidney cells with CpG-ODN. We could demonstrate that treatment with CpG DNA suppressed the basal and the UVBor TNFα-induced interleukin 8 (IL-8) levels of cell culture supernatants. Additionally, it was found that interleukin 6 (IL-6) of stimulated cells was markedly reduced by treatment with CpG-ODN, respectively. Furthermore, we could show that the cytokine suppression is dependent on the number of nucleotides; sequences longer than 12 nucleotides seem to be highly efficient.
To investigate the molecular mechanisms of the CpG-effect we treated epithelial cells with bafilomycin A and chloroquine which both prevent endosomal maturation. This failed to interfere with IL-8 suppression by CpG-DNA suggesting an alternative mechanism.
In conclusion our findings suggest an anti-inflammatory effect of CpG-ODN in epithelial cells indicating that DNA molecules offer a distinct biological activity restricted to the physiological compartment applicated.

P084

Stabilin-1 is Involved in Endocytic and Sorting Processes in Human Macrophages

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Stabilin-1 and stabilin-2 constitute a novel family of fasciclin-domain containing hyaluronan receptor homologs recently described by us (Politz, O. et al, Biochem.J 362, 155-164). Whereas stabilin-1 is expressed in both sinusoidal endothelial cells and in macrophages in vivo, stabilin-2 is absent from the latter. In the present study we analysed the subcellular distribution of stabilin-1 in primary human macrophages. Using flow cytometry, stabilin-1 was shown to be expressed on the surface of interleukin-4/dexamethasone-stimulated macrophages (MΦIL-4/Dex). By immunofluorescense and confocal microscopy we established, that stabilin-1 is preferentially localised in EEA1-positive early/sorting endosomes and in recycling endosomes identified by transferrin endocytosis. Association of stabilin-1 was infrequently seen with p62 lck ligand-positive late endosomes and with CD63-positive lysosomes; but Stabilin-1 was not found in Lamp-1-positive lysosomes. Stabilin-1 was also found in trans-Golgi network, but not in Golgi-stack structures. In vitro studies revealed, that the cytoplasmic tail of stabilin-1, but not of stabilin-2 directly interacts with GGAs - recently discovered adaptors mediating traffic between Golgi and endosomal/lysosomal compartment. Binding to Stabilin-1 in GST pull-down assay was shown for GGA1, GGA2, and GGA3L but not GGA3S. The association was confirmed by demonstration of co-localisation of stabilin-1 with GGA2 and GGA3 in trans-Golgi network of MΦIL-4/Dex. This co-localisation was disrupted by brefeldin treatment and resulted in concentration of stabilin-1 in perinuclear areas. Our results suggest that stabilin-1 is involved in GGA-mediated sorting processes at the interface of the biosynthetic and endosomal pathways; similarly to other GGA-interacting proteins, stabilin-1 may thus function in endocytic and secretory processes of human macrophages.

P085

Mice deficient for the Vitamin D Receptor (VDR -/- mice) show increased local resistance to cutaneous L. major infection

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1,25 dihydroxy vitamin D3 (1,25-VitD3) acts on immune cells (macrophages, t-cells) and is known to have immunmodulatory functions. To analyse these immunmodulatory properties in vivo we infected mice deficient for the Vitamin D Receptor (VDR -/- mice) subcutaneously with Leishmania major (L.m.).
Like C57/Bl6 wildtype mice VDR -/- mice develop a transient local skin lesion. The lesions however were significantly smaller in VDR -/- mice, resolved faster and contained smaller numbers of living parasites than in C57/Bl6 mice. In both mice we observed no spreading of parasites to visceral organs. Living parasites could be found in local lymphnodes. In contrast to the skin however there was no difference in L.m. numbers in lymphnodes.
1,25-VitD3 may affect development of CD4CD25 positive regulatory t-cells (Treg) which are known to inhibit Th1 cells in L.m. skin lesions resulting in an increased parasite survival. In skin lesions of VDR -/- mice we observed lower numbers of Treg than in wildtype mice.
When CD4 positive t-cells were isolated from infected animals and restimulated in vitro with wildtype derived dendritic cellls pulsed with L.m. antigen we did detect a Th1 secretion pattern in both mice. Though 1,25-VitD3 is described as Th2 promoting cytokine in some conditions the absence of the VDR did not result in an increased secretion of Th1 cytokines (like IFNγ) in VDR -/- mice.
Macrophages from VDR -/- mice however produced more leishmanicidal NO after stimulation with IFNγ and L.m. in vitro than macrophages derived from C57/Bl6 mice.
In conclusion VDR -/- mice showed an increased local resistance to L.m. infection which seems to be independent from Th1/Th2 development. The smaller cutaneous L.m. numbers in VDR -/- mice may be caused by an increased production of NO by macrophages lacking the VDR. It is tempting to speculate that local production of 1,25-VitD3 by cells present in the skin (e.g. by macrophages themselves) might result in a deactivation of macrophage killing functions in wildtype mice. Since regulatory t-cells are also known to increase local survival of L.m., the smaller numbers of Treg in VDR -/- mice might also be involved in increased local resistance.

P086

IL-18 induced keratinocyte response contributes to a Th1 like inflammation.

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IL-18, initially termed IFNγ-inducing-factor, has been described to play a role in several inflammatory skin diseases such as eczema and psoriasis. In this study, we aimed to elucidate keratinocytes as potential targets for IL-18 effects. In primary human keratinocytes expression of IL-18Rα as well as responses to IL-18 were determined. In keratinocytes freshly isolated from skin biopsies of lesional atopic dermatitis or psoriasis we observed a markedly higher expression of the IL-18Rα as compared to keratinocytes from normal donors. A marked upregulation was induced in vitro upon stimulation with IFNγ plus TNFα or poly I:C. IL-4 led to downregulation of IL-18Rα. IL-18 induced IP-10 production in freshly isolated keratinocytes from lesional psoriasis as well as in-vitro cultures. IP-10 attracts CXCR3+ Th1 cells.Furthermore, IL-18 up regulated MHC class II expression on keratinocytes. This was of functional significance as verified in coculture experiments with autologous CD4+ T-cells in the presence of superantigen. Autologous T-cells produced significant amounts of IFNγ after coculture with IL-18 induced MHC class II expressing keratinocytes. In conclusion, we have shown for the first time that keratinocytes functionally respond to IL-18 with upregulation of MHC II and production of the chemokine IP-10. These findings further support an important role of IL-18 in inflammatory skin diseases in the epidermal compartment.

P087

Role of IL-10 and TGF-β in the suppression of allergic contact dermatitis by CD4+CD25+ regulatory T-cells

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Introduction: In vitro studies have proven that the suppressive capacity of CD4+CD25+ T-cells requires cell-cell contact, whereas in vivo, suppressive cytokines like IL-10 and TGF-β seem to play an important role in the mechanism of action of CD4+CD25+ regulatory T-cells. We previously demonstrated that the injection of CD4+CD25+ T-cells lead to suppression of inflammatory reactions in different murine in vivo models of hapten-induced contact allergy. Based on the fact, that these injected CD4+CD25+ T-cells were able to suppress without immigrating in the affected tissue, we analyzed the role of anti-inflammatory cytokines associated with CD4+CD25+ regulatory T-cells. Methods: We used two different in vivo models of hapten-induced contact allergy, the well-established model of contact hypersensitivity and the model of skinfold chamber, which permits the visualization of the microcirculatory changes directly at the site of inflammation using intravital fluorescence microscopy in vivo in awake mice. 100μl supernatant of 48h cultured CD4+CD25- or CD4+CD25+ T-cells (10x106 cells/ml medium), or control medium, was i.v. injected before the second hapten-contact. Blocking studies with mAb provided information about the responsible soluble factors in supernatants. Results: Similar to the injection of CD4+CD25+ T-cells, supernatants of these cells suppressed the earswelling-reaction in the model of contact hypersensitivity to approximately 50%. Also, hapten-induced leukocyte-endothelium interactions in the murine model of skinfold chamber were completely blocked. Blocking occurred almost immediately after application of supernatants. Injection of supernatant of CD4+CD25- T-cells or culture medium did not influence the reaction of the allergic contact dermatitis. In vivo administration of anti-IL-10 together with supernatant reversed this effect. Injection of anti-TGF-β had no influence on the suppressive capacity of the supernatant of CD4+CD25+ T-cells. Conclusion: These findings demonstrate that in the suppression of allergic contact dermatitis by CD4+CD25+ regulatory T-cells IL-10 is a crucial factor in vivo.

P088

First steps towards a characterization of cutaneous reinnervation after skin nerve lesion (SNL) in mice

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In order to investigate the influence of T cells and mast cells on peripheral neuroregeneration and reinnervation, we have established and analysed a novel skin nerve lesion (SNL) model.
We have used female B10.PL mice in the telogen stage of the hair cycle (6-9 weeks old) to study the effect of unilateral surgical denervation of dorsal cutaneous nerves (DCNs) in the T3-T12 dermatomes. A 3 cm midline incision was made in the dorsal skin under anaesthesia. The DCNs were exposed under a dissection microscope and DCNs T3-12 on the right side were removed from close to their exit point from the body wall to their entry into the skin. The skin was closed with 9-mm steel wound clips and the completeness of denervation was verified by testing the appropriate skin region for pinch sensitivity. We analysed the immunoreactivity patterns of PGP9.5 (pan-neuronal marker), CD3 (T cell marker) and CD8 (cytotoxic T cell marker). At days 7 and 14 after lesion, immunoreactivity for PGP9.5 was fully absent from the epidermis. Single fibers in the epidermis were found at day 21 after lesion indicating a beginning reinnervation. At day 56 after lesion, the mean epidermal fiber density increased to 30 fibers/microscopic field. Interestingly, also the contralateral, non-denervated side displayed a decreasing fiber density between days 7 and 14 after lesion. No substantial changes in mast cell numbers were detected. Increased numbers of CD3+ and CD8+ T cells were present in the denervated area at days 7 and 14 after lesion.

The skin nerve lesion model offers an attractive tool to analyse the spatio-temporal distribution patterns of nerve fibers and immune cells during reinnervation of murine back skin.

P089

IL-10 modulates expression of members of the B7 family on tolerogenic dendritic cells inducing regulatory T cells

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Previously, we have demonstrated that human IL-10-treated dendritic cells (IL-10DC) are able to induce anergic CD4+ T cells with antigen-specific regulatory activity, partially mediated by CTLA-4 signaling. To analyze the function of members of the costimulatory/coinhibitory family of B7-molecules on DC for the indcution of regulatory T cells, we screened for surface expression of B7-1, B7-2, B7-H2, B7-H3, PD-L1, PD-L2 on human immature, mature and IL-10-treated DC. B7-1 and B7-2 have been demonstrated to activate or inhibit T cell responses, by CD28- or CTLA-4-induced signaling in T cells respectively, whereas B7-H2 (ICOSL), B7-H3, PD-L1 and PD-L2 are known to induce an modulation or inhibiton of T cell responses. DC were generated from peripheral progenitor cells in the presence of IL-4 and GM-CSF for 5 days (immature DC) and subsequently stimulated with a cytokine cocktail containing IL-1-beta, IL-6, TNF-alpha and PGE2 (mature DC), and IL-10 (IL-10DC). FACS analysis demonstrated an upregulated expression of all members of the B7 family on mature DC as compared to immature DC. Treatment of DC with IL-10 strongly reduced the surface intensity of B7-2/CD86. The expression of B7-1(CD80), B7-H3, PD-L1 and PD-L2 was only moderately downregulated, while still being present on >95% of IL-10DC. In contrast to the other members of the B7 family, B7-H2/ICOSL molecules were barely detectable on DC after modulation with IL-10. In addition, blocking experiments with mAb to B7-1 and B7-2 demonstrated that signaling events mediated by B7-2, but not of B7-1, are involved in the induction of anergic regulatory T cells. Thus, these data demonstrate a distinct expression pattern of molecules of the B7 family on immature, mature and IL-10-treated dendritic cells. In contrast to mature DC, IL10-DC are characterized by a reduced cell surface expression of all members of the B7 family, irrespectively of the costimulatory or inhibitory activity. These data suggest that the relative cell surface expression and the binding affinity of the members of the B7 family contributes to the final effect on T cell stimulation.

P090

CD4-specific Abs have the potential to amplify tumor-specific CD8 responses

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We are interested in the identification of strategies aimed at amplifying specific immune responses.
While evaluating the contribution of CD4 cells to the protective effect of certain vaccines, we made the following surprising observation. We found the administration of antibodies (Abs) to CD4 to enable experimental animals to reject inocula of RENCAlacZ tumor cells. While none of 5 control mice was capable of preventing the growth of RENCAlacZ tumors, only 1 of 5 animals injected with Abs to CD4 developed a tumor upon s.c. RENCAlacZ inoculation. The protective effect was long-lived since rechallenge of protected animals 90 days after the first tumor cell implantation did not lead to tumor formation in any of the animals tested. To unravel the effector mechanism involved, we performed CD8 depletion experiments. Elimination of CD8 T cells was found to completely abolish the protective effect of administering Abs to CD4. We thus evaluated the βgal-specific CD8 response in more detail employing ELISPOT analysis. Injection of RENCAlacZ cells into syngeneic BALB/c mice was found to induce low numbers of IFN-γ-producing, specific CD8 cells. This response was maximal only by day 21 after tumor cell inoculation. By contrast, RENCAlacZ-recipients treated with Abs to CD4 were shown to exhibit a pronounced βgal-specific CD8 response (5-10 fold difference) which peaked by day 14. We next evaluated a possible contribution of CD4+CD25+ regulatory T cells to the phenomena observed. Preliminary results for both, the immunological as well as clinical effects seen, are compatible with the notion of the involvement of additional types of regulatory cells.
Taken together, we provide evidence to suggest that the s.c. inoculation of cancer cells induces a CD8 response to antigens expressed by the tumor cells and that this can be significantly enhanced by the administration of Abs to CD4. It remains to be seen whether such a strategy is applicable in a more general sense and whether it can be employed without causing immunosuppression.

P091

Non-complement-fixing IgG4 autoantibodies from bullous pemphigoid patients induce dermal-epidermal separation in cryosections of human skin

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Bullous pemphigoid (BP) is a subepidermal autoimmune blistering disease associated with autoantibodies to the dermal-epidermal junction. Complement and leukocyte activation are major pathogenic events in blister formation of BP. Using cryosections of human skin, we previously demonstrated that BP patients' autoantibodies generate dermal-epidermal separation when co-incubated with leukocytes from healthy volunteers. Circulating autoantibodies in the serum of BP patients mainly belong to IgG1 and IgG4 subclasses. It has been suggested that IgG1 antibodies with their strong complement- and leukocyte-activating capacity are the pathogenically relevant autoantibodies in BP. In contrast, non-complement-fixing IgG4 autoantibodies, that poorly bind to leukocytes, were thought not to be primarily involved in blister formation. However, the subclass of pathogenic IgG autoantibodies in BP sera has not yet been clearly demonstrated. Therefore, in this study, we purified both IgG1 and IgG4 autoantibodies from BP patients' serum and analyzed their blister-inducing potential in our cryosection assay. Purification of IgG subclasses from BP sera was performed using monoclonal antibodies against IgG1, IgG2, IgG3, or IgG4 which were immobilized on an affinity matrix. Purified IgG1 and IgG4 preparations were brought to the same reactivity against the epidermal basement membrane by indirect immunofluorescence microscopy as compared with the subclass reactivity of the crude BP sera. Subsequently, purified antibodies were incubated with cryosections of human skin in the presence of leukocytes. As expected, IgG1 autoantibodies fixed complement to the dermal-epidermal junction and induced subepidermal splits in this experimental model. Purified IgG4 did not fix complement, but, interestingly, like IgG1, recruited and activated leukocytes to the basement membrane and induced dermal-epidermal separation. Our results demonstrate that, in addition to IgG1, IgG4 autoantibodies are able to activate leukocytes and point to a hitherto less recognized function of IgG4. Moreover, for the first time, we clearly demonstrate that IgG4 autoantibodies from BP patients have the capacity to induce dermal-epidermal separation. These findings should also be important for the treatment of BP, e.g., when choosing appropriate affinity matrices for extracorporeal immunoadsorption techniques which have now become available.

P092

Peripheral CD8+ T cell tolerance against melanocytic self antigens in the skin is regulated in two steps by CD4+ T cells and local inflammation: Implications for the pathophysiology of vitiligo

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Experimental evidence has suggested a role for CD8+ cytotoxic T lymphocytes (CTL) in the pathophysiology of vitiligo, a pigmentation disorder with focal loss of melanocytes in the skin. The discovery of tyrosinase-related protein 2 (TRP2) as a model melanocytic self antigen recognized by CD8+ CTL in C57BL/6 mice allowed us to analyse the requirements for CD8+ T cell-mediated autoimmune destruction of melanocytes in an experimental model. Using two different genetic methods for the induction of cellular immunity in vivo, gene gun bombardment of the skin and injection of recombinant adenovirus, we show that peripheral tolerance of CD8+ T cells recognizing a single TRP2-derived H2-Kb-binding peptide is regulated in two steps. In the induction phase, stimulation and expansion of TRP2-specific CD8+ T cells in vivo depends on CD4+ T cell help. In the effector phase, autoimmune destruction of melanocytes in the skin depends on local inflammation. Our results suggest that accidental stimulation of CD8+ CTL recognizing MHC class I-binding peptides derived from melanocytic proteins in the context of an inflammatory skin disease may play an important role in the pathophysiology of vitiligo.

P093

NKG2D/ULBP-1 interaction for DC-mediated T cell activation

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The immune system can be divided into an innate and adaptive immunity. The systems, however, are not separately evolved. In this regard, NKG2D first identified on NK cells and classified as activating NK receptor is also an important receptor for CD8+ T cells. The analyses of the role of NKG2D and its ligands, i.e. ULBPs and MICs, so far focused on immune cell-target cell situations, due to the expression of NKG2D ligands on infected, stressed or transformed cells. Here, however, we analyzed NKG2D and ULBP-1 expression on immune competent cells of melanoma patients. To this end, ULBP-1 expression on mature dendritic cells can be detected both in situ in the T cell areas of lymph nodes as well as in vitro after artificial maturation. Notably, ULBP-1 mRNA expression was tightly regulated in the process of DC maturation. FCM analysis further demonstrated that although NKG2D is partially expressed on all analyzed T cell subsets from peripheral blood, in vitro stimulation of T cells resulted in an up-regulation of NKG2D on proliferating T cells. Most important, in situ analysis of the sentinel lymph nodes of primary melanoma revealed the expression of NKG2D on melanoma associated antigen specific T cells, but not on T cells recognizing a viral peptide. Additionally, we observed a co-localization of NKG2D and activation marker expression. Thus, our results imply an important role of NGK2D-ULBP-1 signaling for newly initiated primary or reinitiated memory T cell immune responses.

P094

In vivo CD40 ligation promotes the therapeutic efficacy of a genetic melanoma vaccine by promoting both innate and adaptive immune responses

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Mechanisms maintaining peripheral tolerance to self antigens present a major obstacle for the development of antigen-specific melanoma vaccines, presumably because self antigens are not able to stimulate a CD4 T-helper response. We recently reported that effective stimulation of melanoma-specific CD8+ T cells in mice vivo could be obtained by genetic immunization with a fusion protein providing linked CD4+ T-cell help. Here show that protection against melanoma cells can be mediated by CD8+ T cells specific for a single H2-Kb-binding peptide epitope derived from the melanosomal protein TRP2. We confirm that in vivo stimulation and expansion of melanoma peptide-specific CD8+ T cells strictly depends on the presence of CD4+ T cell help during the priming but not the effector phase. Furthermore, we found that antibody-mediated in vivo CD40 ligation can substitute for CD4 T cell help and increase the efficacy of genetic melanoma immunization as effectively as linkage with immunogenic proteins. Most importantly, CD40 ligation was able to substantially enhance the therapeutic efficacy genetic immunization by stimulating both the innate and the adaptive arm of the immune system. Our results help to elucidate the requirements for effective in vivo stimulation of cellular immunity to melanoma and provide important information for the translation of this strategy in future clinical applications.

P095

Evaluation of hsp70 as a molecular adjuvant for genetic melanoma immunization in mice

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The melanosomal enzyme tyrosinase-related protein 2 (mTRP-2) is expressed by melanocytes and most melanoma cells. It represents an attractive, clinically relevant antigen for the experimental development of melanoma immunotherapy in mice because it can be recognized by MHC class I-restricted cytotoxic T cells. Many immunization strategies targeting mTRP-2 have been experimentally developed including vaccination with recombinant adenoviruses. Mechanisms maintaining peripheral tolerance against self-antigens limit the induction of a strong immune response against TRP2. Recently, heat-shock proteins have become attractive molecular adjuvants capable of enhancing weak immune responses by increasing antigen presentation and stimulating the production of cytokines. In the present study, we constructed recombinant adenoviruses encoding the melanoma antigen TRP-2 or the artificial model antigen EGFP.OVA fused to human heat-shock protein 70 (Hsp70). The expression of these fusion proteins was confirmed in vitro. Currently, we are evaluating the efficacy of these recombinant adenoviruses in vivo in the murine B16 melanoma model. These experiments will provide information whether genetic vaccines consisting of self-antigens directly fused to Hsp70 can be successfully applied for antigen-specific immunotherapy of melanoma and may provide a scientific basis for the translation of this strategy in future clinical trials.

P096

The role of IFNα for genetic immunization targeting dendritic cells in vitro and in vivo

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IFNα is primarily induced during certain viral infections and bridges innate and adaptive immune responses. IFNα is able to activate DC and NK cells and supports proliferation, differentiation and effector functions of antigen-specific cytotoxic T cells. In the present study we evaluated the role of IFNα on the induction of cellular immune responses following genetic immunization of C57BL/6 mice. We constructed recombinant adenoviruses simultaneously encoding IFNα and the artificial model antigen EGFP.OVA or the melanoma antigen TRP-2 linked via a viral internal ribosome entry site. Expression of IFNα in cultured dendritic cells limited antigen synthesis and promoted maturation and antigen presentation as evidenced by an increase of MHC and costimulatory molecules on the cell surface. Currently, we are evaluating whether coexpression of IFNα also is able to promote maturation as well as antigen-processing and -presentation of dendritic cells directly in vivo and enhance the induction of CD8+ T cells. Furthermore, we will evaluate whether IFNα can circumvent peripheral tolerance against the self antigen TRP2. These results will help to elucidate the role of IFNα in the regulation of cellular immunity in vivo.

P097

A dissociated Vitamin D3 Analog Mediates Potent Immunomodulatory Effects on Immune Cells

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1fÑ,25-Dihydroxyvitamin D3 (Calcitriol), the biologically active form of vitamin D3 has potent immunomodulatory activity, however its clinical use as an immunomodulator is limited due to its prominent role in calcium homeostasis. Recently, we described a novel vitamin D3 analog, ZK 191784 which exhibit immunomodulatory activity in a murine model of DNFB-induced allergic contact dermatitis also in non-hypercalcemic dosages. The aim of the present study was to analyze the underlying mode of action behind the immunomodulation. In order to investigate whether inhibition of edema formation induced by ZK 191784 is based on a cellular component (e.g. activation of regulatory immune cells) and can be transferred to recipient animals adoptive transfer experiments have been performed. As shown in these studies, transfer of immune cells from DNFB-primed and DNFB-challenged donor mice treated with ZK 191784 results in a significant edema inhibition by approx. 60% in recipient mice after challenge with DNFB. A similar degree of edema inhibition were obtained in recipient mice with transfer of immune cells from Cyclosporin A-treated mice. In vitro kinetic experiments on human PBMC showed that a short term incubation of 15 minutes caused a maximum downregulation of HLA-DR and a maximum upregulation of CD14 on monocytes as analyzed 48 h after culture setup. Other markers such as ICAM-1 and CD80 were also downregulated by ZK 191784 but required a longer incubation period. These findings demonstrate that ZK 191784 has potent immunomodulatory effects on immune cells. From the in vitro kinetic experiments we conclude that ZK 191784 potently suppresses HLA-DR and co-stimulatory markers on antigen presenting cells and that even a short exposure of these cells (here: monocytes) to ZK 197184 is sufficient to exert maximum effects on HLA-DR expression levels. Since reduced expression of these molecules limits T cell responses these effects exerted by short exposure to ZK 191784 may also be relevant in the in vivo situation.

P098

Alphavirus-based genetic immunization circumvents peripheral tolerance against the melanocytic antigen TRP2 and promotes autoimmune vitiligo in C57BL/6 mice

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CD8+ cytotoxic T lymphocytes (CTL) recognizing the model melanocytic self antigen tyrosinase-related protein 2 (TRP2) are able to destroy melanocytes in the hair follicle of C57BL/6 mice leading to vitiligo-like fur depigmentation. Stimulation of TRP2-specific CD8+ CTL in vivo is limited by mechanisms maintaining peripheral tolerance against self-antigens. In this study, we investigate whether a gene gun-based immunization protocol using a Sindbis virus-based expression construct is able to circumvent TRP2-specific CD8+ T cell tolerance. cDNA coding for TRP2 was inserted into the plasmid pSIN and antigen expression confirmed in transiently transfected 293 cells using immunofluorescence and western blot. Subsequently, groups of mice were immunized with pSIN-mTRP2 or appropriate controls. To date, vitiligo-like fur depigmentation has been observed in a number of C57BL/6 mice immunized with pSIN-mTRP2. Currently, we are investigating the induction of TRP2-specific CD8+ T cells and antibodies and are immunizing CD8 knockout mice to further elucidate the role of CD8+ T cells. Our results provide evidence that alphavirus-based genetic immunization can circumvent peripheral tolerance against the melanocytic antigen TRP2. Subsequent experiments will have to elucidate the cellular and molecular mechanisms involved in the efficacy of this novel immunization strategy.

P099

Enhanced type I interferon signaling promotes a Th1 biased inflammation in cutaneous lupus erythematosus via induction of IP10/CCL10 and recruitment of CXCR3+ lymphocytes

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Recent studies suggested that type I interferons (IFN) play a role in the pathogenesis of lupus erythematosus (LE), an autoimmune disease with unknown etiology. Natural interferon producing plasmacytoid cells have been demonstrated in cutaneous LE (CLE) lesions, along with elevated levels of IFNα mRNA. We hypothesized that local production of type I IFNs in CLE induce a Th1 biased inflammation via induction of IFN-inducible chemokines such as IP10/CXCL10 leading to the recruitment of chemokine receptor CXCR3 expressing T cells into skin lesions.
Skin biopsies were taken from 21 patients suffering from different types of active cutaneous LE and analyzed for the expression of MxA, a protein specifically induced by type I interferons, the IFN-inducible protein IP10/CXCL10, and the chemokine receptor CXCR3, characteristic for Th1 cells, by immunohistochemistry. Additionally, peripheral CD4+ and CD8+ T cells were investigated for the expression of MxA and CXCR3 by flow cytometry. Cell culture analyses were performed to investigate the chemokine-production of keratinocytes after stimulation with IFNα.
Cutaneous LE lesions were characterized by strong expression of MxA indicating the induction of localized type I IFN signaling in the skin. Large numbers of infiltrating CXCR3 positive lymphocytes were detected in CLE skin lesions, which closely correlated with the lesional MxA expression (epidermis: Spearmans $r=0.56$, $p<0.001$; dermis: $r=0.82$, $p<0.001$). Intracellular MxA levels of circulating CD4+ and CD8+ T cells were significantly enhanced in active CLE patients. The percentage of peripheral T cells expressing CXCR3 was significantly decreased in specific CLE subtypes. Expression of IP10/CXCL10 in the epidermis links type I IFN signaling and recruitment of CXCR3+ T cells. Cell culture analyses confirmed the induction of the expression of IP10/CXCL10 in keratinocytes after stimulation with IFNα.
Our results suggest an important role for type I interferon signaling in the pathogenesis of cutaneous lupus erythematosus. We propose, that type I IFNs induce a Th1 biased inflammatory immune response, with recruitment of CXCR3 expressing T lymphocytes into the skin via IP10/CXCL10.

P100

THE RECEPTOR FOR INTERLEUKIN-17E IS INDUCED BY TH2 CYTOKINES IN ANTIGEN PRESENTING CELLS

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Interleukin (IL)-17E (IL-25) is a recently identified cytokine capable to induce Th2-associated cytokine production (IL-5, IL-13) and Th2-type pathologies in animal models. The IL-17E responsive cell population in vivo was described to be a furthermore uncharacterised non-T, non-B splenic accessory cell. Despite the identification of IL-17BR as the receptor for IL-17E, the cell population expressing IL-17BR has hitherto not been identified.
To test whether APC2 express IL-17BR, we analysed the expression of IL-17BR mRNA in human MΦs and DCs using real time RT-PCR analysis. The results showed an about 10 fold higher IL-17BR expression level in MΦ stimulated by IL-4 (MΦ2IL4) as compared to MΦ stimulated by IFNγ (MΦ1IFNγ). The presence of IL-17BR protein was confirmed by Western blot analysis. In order to test if other Th2 associated cytokines (e.g. IL-10, IL-13 and TGFβ) or glucocorticoids (GC) influence the expression of IL-17BR in a manner similar to IL-4 we analysed IL-17BR expression in MΦ stimulated by IL-10, IL-13, TGFβ or dexamethasone and by the combinations of these cytokines with IL-4. Real time RT-PCR analysis showed that IL-13 alone has a similar effect on IL-17BR as IL-4, but did not enhance the inducing effect of IL-4. Dexamethasone inhibited the expression of IL-17BR in IL-4 stimulated MΦ. IL-10 did not have any significant effect, while TGFβ significantly increased the expression of IL-17BR when used in combination with IL-4. Surprisingly, TGFβ alone exerted only a minor stimulating effect. Similar results were obtained for IL-10-induced human myeloid regulatory dendritic cells (DCreg-IL-10). Additionally, soluble form of IL-17BR was found in MΦ2 on the mRNA level.
These results indicate that IL-17BR expressing APC2s may mediate the development of the IL-17E-mediated immunological reaction patterns observed in vivo.

P101

Senescent BALB/c mice are able to develop resistance to L.major

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Resistance to infection with L.major in mice requires a Th1 response as well as efficient antigen presentation by dendritic cells and NO production by macrophages. Senescence is associated with an declined resistance to several infections, partially due to dysregulations of macrophage functions such as NO production. We have formerly shown that these cellular deficiencies did not influence resistance in C57Bl/65 mice to L.major. As senescence was also shown to be associated with decreased Th2 responses towards certain antigens (e.g. helminths), and as we observed less footpad swelling in ageing BALB/c mice after L.major infection, we further analysed influence of ageing on this particular immune response. Infection of BALB/c mice not only revealed lower parasite numbers in footpads of 18-month old (senescent) than in 2-month old (young) mice, but also healing of ulcers in 40% of mice, as well as reduced parasite dissemination and a Th1 cell-like response. These changes were associated with spontaneous release of IL-12 by bone-marrow derived macrophages from aged, but not from young mice.
As exogenous factors such as microbial exposure can influence immune responses we next infected old mice raised in conditions free of specific pathogens (SPF). They revealed neither resistance nor a Th1 response while their macrophages still spontaneously released IL-12. Thus, senescence and IL12 are necessary, but not sufficient for turning susceptibility into resistance.
Next we performed detailed microbiological analysis which revealed that conventionally kept mice, but not SPF mice were infected with Murine Hepatitis Virus. This infection had been shown to favour a Tc1- or Th1-like response.
Our model has striking parallels to observations in Th2-mediated type I allergies in humans which are also reduced after continuous microbial exposure and during senescence.
We conclude that for this reversal of the immune response senescence is the premier requirement which needs to be completed by another mandatory event such as microbial stimulation. One of the age-, but not environment-related factors is spontaneous release of IL-12 by macrophages, while confrontation with MHV presents an environment-related difference, with both having the potential to support a Th1 response

P102

Interleukin-4 and dexamethasone counterregulate ECM remodelling and phagocytosis in type 2 macrophages

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Alternatively activated macrophages (MΦ2) are induced by Th2 cytokines and by glucocorticoids (GC), and can be distinguished from classically activated effector macrophages (MΦ1) on the basis of their anti-inflammatory properties. In addition, MΦ2 are involved in Th2/Th1 skewing, enhance antigen uptake and processing, and support tissue remodelling and healing. To systematically elucidate the heterogeneity of MΦ2 population we analysed a number of genes involved in ECM remodelling, inflammation and phagocytosis in MΦ2 populations generated with IL-4 or GC. By Real-Time PCR we demonstrated that the extracellular matrix (ECM) component tenascin-C is stimulated by IL-4 while it is suppressed by dexamethasone. The ECM remodelling enzymes MMP-1, -12, and tissue transglutaminase showed a similar regulation pattern. FXIIIa, another putative MΦ2-associated transglutaminase, was synergistically regulated by IL-4 and GC. ELISA analysis revealed that the production of MΦ2-associated chemokines AMAC-1, MCP-4, or TARC was induced by IL-4 and modulated by GC. Phagocytosis of opsonized and non-opsonized particles was stimulated by GC, while IL-4 had only a modulatory effect, what may be partially explained by the expression pattern of hMARCO, a scavenger receptor for non-opsonized particles, that was strongly and selectively induced by GC. In conclusion, stimulation of MΦ with IL-4 and GC regulate antagonistically the expression of ECM remodelling-related molecules and phagocytosis of opsonized and non-opsonized particles.

P103

Sweat of patients with atopic dermatitis contains a reduced amount of dermcidin-derived antimicrobial peptides and shows a decreased antimicrobial activity in vivo

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Antimicrobial peptides (AMP) are an integral part of the epithelial innate defense system. Dermcidin (DCD) is a recently discovered AMP with broad spectrum of activity and no homology to other known AMPs. DCD is constitutively expressed in human eccrine sweat glands and transported via sweat to the epidermal surface. In atopic dermatitis (AD) recurrent bacterial or viral skin infections and colonisation with S. aureus are characteristic features indicating compromised innate immune mechanisms.
To analyze whether sweat of patients with atopic dermatitis (AD) contains a reduced amount of DCD-peptides we performed semiquantitative and quantitative analysis of several processed DCD peptides in sweat of AD patients and healthy subjects using surface-enhanced laser-desorption ionisation (SELDI) and ELISA. Furthermore, we analyzed whether sweat of AD patients exhibits a reduced antimicrobial activity in vivo.
Using SELDI we found that the proteolytic pattern of DCD-derived peptides in sweat were similar between healthy and atopic subjects. In contrast, using semiquantitative SELDI-analysis we demonstrated that the amount of DCD-derived peptides in sweat of patients with atopic dermatitis is significantly reduced. In addition, compared to atopic patients without previous infectious complications AD patients with a history of bacterial and viral skin infections were found to have significantly less of the DCD-peptides in their sweat. Furthermore, we confirmed a specific decrease of DCD in sweat of AD patients using ELISA. To analyze whether the reduced expression of DCD in sweat of AD patients correlates with a decreased innate defense we determined the antimicrobial activity of sweat in vivo. We showed that in healthy subjects sweating leads to a reduction of bacterial skin colonisation, however, not in patients with atopic dermatitis.
These data indicate that reduced expression of DCD in sweat of patients with atopic dermatitis may contribute to the high susceptibility of these patients to skin infections and to altered skin colonisation.

P104

ACTIVE LFA-1 ON DC IS INHIBITORY FOR FULL T CELL ACTIVATION

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The beta2 integrin LFA-1 is important for transendothelial migration of leukocytes as well as for T cell activation during antigen presentation. To determine the relevance of LFA-1 for antigen presentation of DC, we investigated bone marrow-derived DC (bmDC) from CD18-deficient (-/-) mice, which lack all functional beta2 integrins, in vitro. Despite the abundance of LFA-1 on murine wild type bmDC, functional studies measuring contact times of DC and T cells in collagen gels as well as MLR and cytokine production of T cells revealed no impact of LFA-1 on bmDC for T cell activation, compared to CD18-deficient bmDC. To study how LFA-1 on bmDC is inactivated, we examined a mechnism that has been shown to inactivate LFA-1 in human monocyte-derived DC. There, Cytohesin-1, a cytosolic protein that has been shown to activate LFA-1 when it aggregates CD18 at the inner membrane, is sequestered by Cytip, and kept into the cytosol, thereby rendering LFA-1 inactive. Here, using confocal microscopy and Western Blot we show that the same mechanism applies in murine bmDC. This mechanism seems to be DC-specific as it is not found in T cells and quiescent B cells due to the lack of Cytip expression. To examine the biological relevance of inactive LFA-1 on bmDC we used cations Mg2+ and Ca2+ to activate the integrin LFA-1. Indeed, Mg2+ and Ca2+ activated LFA-1 on bmDC shown by adhesion of DC to the LFA-1-specific ligand ICAM-1. When employed into MLR bmDC with active LFA-1 showed impaired capacity to stimulate T cell activation measured by proliferation and cytokine production of T cells. Thus, we speculate that inhibition of LFA-1 on bmDC is an active process and might be necessary to achieve full T cell activation.

P105

Induction of OVA expression in the skin under homeostatic conditions

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Cutaneous immune surveillance seems to be important in host defence mechanisms. However, the "surveillance function" of the skin has never been definitively proven to be operative in vivo under physiological conditions. Depending on the cellular and molecular microenvironment, immune responses generated by skin associated lymphoid tissues can lead not only to protective immunity against pathogens, but also to tolerance.
To study immune responses to a de novo expressed antigen in the skin under homeostatic conditions in vivo, we have generated transgenic mice expressing the surrogate antigen ovalbumin (OVA) in a Cre-inducible manner in keratinocytes (K5-loxP-YFP-loxP-OVA-IRES-CFP). Expression of OVA is either induced around embryonic day 14.5 (E 14.5) with a constitutively expressing K5-Cre transgenic line or in adult mice with an Tamoxifen (TX) - inducible K5-CreTM transgenic line. Several transgenic lines have been obtained, which, in an uninduced state, express the yellow fluorescent reporter gene (YFP) but not the OVA gene in epidermal cells. After TX application and Cre-mediated excision of YFP, OVA can be induced, as evidenced by the expression of the OVA257-264 peptide SIINFEKL on keratinocytes by FACS analysis. Moreover, these keratinocytes are able to stimulate the TCROVA specific T-cell hybridoma cell line 4B10 to produce IL-2. Although OVA is efficiently expressed and presented, the transgenic mice do not develop any obvious disease symptoms like hair or weight loss. Moreover, no differences in thymic and peripheral T-cell numbers can be observed after K5CreTM-mediated OVA expression. To monitor CD8+ T-cell mediated immune responses the transgenic mice were crossed with OT1 mice expressing a TCR specific for SIINFEKL-H2kb on all T-cells. No differences in the numbers of antigen specific T-cells could be seen in the periphery, although we find activation of antigen specific T-cells as indicated by upregulation of CD44. Interestingly, if OVA expression is induced at E 14.5 with the K5-Cre line, transgenic mice are smaller and display skin and hair abnormalities.
Taken together our results suggest that depending when a de novo antigen is induced in the skin it can lead to different immunological responses. Whether this is dependent on the antigen concentration is currently under investigation.

P106

Secreted Staphylococcal Products Cause Early Induction of Defence Molecules in Human Keratinocytes.

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Staphylococcus aureus is one of the major skin-infecting bacteria which often causes formation of pus and abscesses. Since staphylococci are commonly found on skin we asked whether there are defence mechanisms fighting staphylococcal infections. We followed the hypothesis, that S. aureus secretes pathogen-associated molecules (PAMs), which may activate keratinocytes to produce proinflammatory mediators and innate defence effector molecules. In previous studies we have shown, that S. aureus cultured under static conditions secrete such PAMs, which we could partially characterize. This study focuses on spectrum and time course of epithelial responses to S. aureus PAMs. We assayed keratinocytes (HaCaT, primary human keratinocytes) incubated with S. aureus supernatants between 2 and 8 hours for mRNA expression of different defence molecules (Real-Time PCR). As examples for innate defence effector molecules we assayed mRNA of the in vivo-relevant S. aureus- killing antimicrobial protein RNase7 and the iron-depleting lipocalin NGAL. As representatives of inflammatory molecules we tested for the proinflammatory cytokines IL-8, IL-6 and TNF-alpha and of the chemokine MIP-3alpha. Already after 2 h of incubation IL-8, IL-6 and TNF-alpha mRNA expression was markedly increased in HaCaT cells as well as in primary keratinocytes. After 4 hours of incubation mRNA levels of the chemokine MIP-3alpha was elevated in HaCaT cells and primary human keratinocytes as well as RNase7 mRNA. The mRNA of the lipocalin NGAL also increased after 4 h of stimulation.
Our results indicate that skin keratinocytes employ multiple rapid strategies against beginning S. aureus infections: Staphylococci can be directly killed by production of antimicrobial proteins as RNase7. Bacterial nutrients may be depleted from the bacterial environment by keratinocyte-derived NGAL that binds iron essential for bacterial growth. Activation of immature dendritic cells by MIP-3alpha links innate and adaptive immune system. Proinflammatory cytokines trigger inflammatory responses which may lead to formation of pus typical for staphylococcal infections by attracting and activating neutrophilic granulocytes. Our further investigations will reveal the different mechanisms and PAMs that elicit the various lines of defence against S. aureus infections.

P107

Deletion of autoreactive T cells does not protect from autoimmune disease

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Transfer experiments have shown that autoreactive, interferon (IFN-)γ producing CD4+ Th1 cells have the potential to induce inflammatory autoimmune diseases in healthy mice. Yet, evidence is lacking that deletion of these autoreactive inflammatory T-cells prevents from autoimmune disease. For our studies we used a mouse model of autoimmune pancreatitis: 415-EL-I-E C57BL/6 transgenic mice express a `non-self# MHC II I-E exclusively on the exocrine pancreas. T cell reactivity to this "autoantigen" is associated with T cell receptors containing vβ5 chains. In 415-EL-I-E mice 4% of the CD4+ T cells express the vβ5 chain. When compared with syngenic C57BL/6 mice TCR-vβ5+ CD4+ T cells are not clonally deleted and proliferation as well as IFN-γ production of vβ5+CD4+ T cells are fully preserved. While in some animal facilities 415-EL-I-ExC57BL/6 mice remain healthy in others they develop inflammation and destruction specifically of the exocrine pancreas. Stable blood glucose levels also demonstrate that the endocrine pancreas remains healthy.
To prevent autoimmune pancreatitis by deletion of the autoreactive vβ5+CD4+ T cells, we crossed 415-EL-I-E mice to 107-I-E mice, expressing the "MHC class II I-E autoantigen" under the class II-promoter also on B cells, macrophages, dendritic cells and thymic epithelium. Though deletion was successful in 415-EL-I-Ex107-I-E F1 mice and vβ5+ T cells were reduced more than ten fold and did not proliferate, deletion of autoreactive vβ5+ T cells did not prevent pancreas destruction in 415-EL-I-Ex107-I-E mice.
To study the role of cross-reacting T cells we further crossed 415-EL-I-E mice to T-cell deficient C57BL/6 RAG-2-/- mice. Absence of T cells in 415-EL-I-ExRAG-2-/- was shown with flow-cytometric analysis of the lymphatic tissues. Surprisingly, pancreas destruction in 415-EL-I-ExRAG-2-/- mice was even enhanced, suggesting that at least under certain conditions, autoreactive T cells may exert a protective role.

P108

Gene regulation of the skin-derived antimicrobial peptide human beta-defensin-3 (hBD-3)

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Human beta-defensin-3 (hBD-3) is an inducible peptide antibiotic originally isolated from lesional psoriatic skin. HBD-3 exhibits a wide range of antimicrobial activity against both gram-positive and gram-negative bacteria, including multi-drug resistant Staphylococcus aureus. The expression of hBD-3 is induced by proinflammatory cytokines, bacteria and Phorbol-12-Myristate-13-Acetate (PMA). In primary keratinocytes we observed the highest hBD3 gene induction after 24 h of incubation with IFN-γ, supernatants of mucoid Pseudomonas aeruginosa (PA) and PMA.
Since little is known about the signal transduction pathways leading to hBD-3 gene induction we investigated the transcription factors involved in PA and PMA mediated gene induction of hBD-3. Primary keratinocytes were transfected with different hBD-3-promoter driven luciferase reporter plasmids and subsequently stimulated with PA or PMA. Activation of the gene reporter depended on the length of the included hBD-3 promoter region: Strong activation was achieved with a promoter fragment containing potential binding sites for the transcription factors NF-κB and AP-1. Stepwise deletion of promoter segments containing potential NF-κB and AP-1 binding sites resulted in decreased luciferase activity suggesting that these transcription factors play an important role in PA- and PMA-mediated hBD-3 induction.
To further analyse the influence of NF-κB and AP-1 on hBD-3 gene induction, we investigated the effect of the NF-κB-inhibitor Helenalin and inhibitors of the three AP-1 inducing MAP-kinase cascades (ERK1/2-inhibitor PD 98059, JNK-inhibitor SP600125 and p38-MAPK-inhibitor SB202190). Realtime-PCR studies following stimulation of primary keratinocytes with PA or PMA and co-incubation with the specific pathway inhibitors confirmed the involvement of NF-κB and AP-1: Inhibition of NF-κB reduced PA- and PMA-mediated hBD-3 induction by 80-95%. Inhibition of ERK-1/2- or p38-MAP-kinase abolished PMA-mediated hBD-3 induction and decreased PA-mediated hBD-3 induction by approximately 70% and 85%, respectively. Inhibition of JNK-MAP-Kinase reduced hBD-3 induction by approximately 45%.
Our data indicate that NF-κB and AP-1 may play an important role in the regulation of hBD-3 gene induction.

P109

Melanoma vaccine development: Comparative analysis of maturation and loading strategies with regard to dendritic cell phenotype and T cell stimulatory capacity by use of an in vitro culture platform

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In recent years dendritic cells (DCs) have become popular candidates in cancer vaccine development because of their crucial role in inducing T-cell responses. However, DC-based clinical studies greatly differ in their protocols for DC generation/maturation and their efficacy in treating established tumors has to be improved. We therefore systematically analysed the T cell stimulatory capacity of clinical grade DCs after maturation and antigen loading by various protocols. Monocyte derived DCs maturated by an inflammatory cytokine cocktail (iCC), CD 40 ligation (CD40L), a combination of iCC and CD40L or immature DCs were loaded with tetanus toxoid (model antigen) or apoptotic bodies versus necrotic cell material of an allogenic malignant melanoma cell line (Mel 526) and were then analysed with the help of an in vitro culture platform for their ability to induce and expand antigen reactive IFN-gamma producing autologous T cells. The resulting phenotype of DCs (fluorescence microscopy and flow cytometry), the time of DC survival under wash out conditions (tryphan blue exclusion) and the capacity to induce antigen reactive T cells (INF-gamma ELISPOT) were comparatively analysed. The highest level and stability of phenotypical DC maturation was found after iCC plus CD40L (85% ±7% double positive staining for CD80 and CD83) and closely correlated with an increased time of cell survival (42% ±8% cell survival after 3 days of cytokine wash out culture). DCs maturated by iCC or CD 40L alone were able to induce the highest level of antigen reactive INF-gamma producing T cells after loading with tetanus toxoid or melanoma antigens. Though with interindividual differences, apoptotic bodies were preferable over necrotic cell material for DC loading (IFN-gamma ELISPOT counts up to 5 times higher for apoptotic bodies).

P110

Fumarates induce a DC2 phenotype in dendritic cells that establishes protective Th2 responses

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Inflammatory organ-specific autoimmune diseases such as psoriasis or multiple sclerosis are considered to be T cell mediated autoimmune diseases dominated by interferon (IFN-)γ producing CD4+ T cells (Th1). As fumaric acid esters (FAE) are highly effective in the treatment of psoriasis and capable of inducing a Th2 phenotype in human T cells we analyzed the effect of various FAE on dendritic cell (DC) functioning. Incubation of antigen presenting cells (APC) or DC with either monomethyl fumarate (MMF) or dimethyl fumarate (DMF) had little or no impact on APC survival. Yet, when restimulated in vitro, MMF- or DMF-incubated APC produced less than 5% to 30% of IL-12, as compared to control populations. In some experiments IL-12 production was completely abrogated. In order to address the functional phenotype of theses APC, we used MMF- or DMF-incubated APC to prime naive ovalbumin (OVA)-specific CD4+ T cells with OVA Peptide. Following stimulation and 10 days in vitro culture we restimulated these CD4+ T cells with fresh APC and OVA peptide for cytokine production. Data was compared to CD4+ T cells primed with shame treated APC. Under these conditions, sham treated APC primed CD4+ T cells to become a Th1 phenotype while MMF- or DMF-treated APC induced a Th2 phenotype in naive CD4+ T cells. As FAE also promoted a Th2 development during in vivo priming of CD4+ T cells and protected against Th1-mediated autoimmune disease, our data suggest that FAE may protect and even improve inflammatory autoimmune diseases through the induction of `protective DC2#.

P111

Gene expression of nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins in primary keratinocytes

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Human keratinocytes have the capacity to initiate a defense response by the release of antimicrobial proteins and proinflammatory cytokines following the detection of microorganisms and their products (so-called "pathogen-associated molecules or patterns, PAMPs"). Detection of these PAMPs is mediated via specific receptors (so-called "Pattern Recognition Receptors, PRRs") which are able to recognize specific PAMPs. It has been shown that various Toll-like receptors, which are involved in the detection of microbes in the extracellular compartment, are expressed in keratinocytes suggesting that they function in keratinocytes to recognize PAMPs. Recently, a novel class of intracellular receptors have been implicated in the recognition of specific PAMPs. These NBS-LRR proteins (for nucleotide-binding site and leucine-rich repeat), are involved in intracellular recognition of microbes and their products (e.g. peptidoglycan). To gain more insight into the role of these putative sensors of microbial products in the innate immune response of human skin we analysed the gene expression of NBS-LRR proteins in keratinocytes using conventional RT-PCR as well as real-time PCR. Intron-spanning primer pairs were designed to differentiate between cDNA and contaminating genomic DNA. Distinct gene expression of 7 NBS-LRR proteins (out of 20 tested) were detected in primary foreskin-derived keratinocytes. Among them were Nalp1, Nalp2, Nalp 10, NOD1, NOD2, NOD9 and to a minor degree NOD27. Whereas most of the genes were not influenced by stimulation with a mucoid Pseudomonas aeruginosa, NOD2 and Nalp10 were slightly induced.
We conclude that keratinocytes express a distinct subset of NBS-LRR proteins. Further studies are now in progress to elucidate whether these putative intracellular PRRs initiate a defense response in keratinocytes upon sensing of microbial products.

P112

IMMUNOBLOT ANALYSIS OF VARIOUS PROTEIN FRACTIONS OF BORRELIA BURGDORFERI, INCLUDING VARIABLE MAJOR PROTEIN - LIKE SEQUENCE EXPRESSED DURING THE COURSE OF ERYTHEMA MIGRANS DISEASE

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To examine the immune response against different proteins of Borrelia burgdorferi (Bb) in erythema migrans (EM) patients before and after therapy by an immunoblot test (IB) including the Variable major protein-like sequence, expressed (VlsE).
In 50 adult EM patients, Bb IgG and IgM antibodies were analyzed in a median of 5 serum samples consecutively obtained before and during a median of 513d (range, 414-1185d) after therapy by recombinant IB. The antigens used in the IgG IB were Outer surface protein (Osp) 17, p41i, OspC, VlsE, p39, p58, p100. In the IgM IB p41i, OspC, p39, and p100 were used. All serum samples were also tested by a standard IgG and IgM ELISA. The IgG IB gave positive results in 50% before and in 57% directly after therapy (ELISA: IgG 22% and 25%, respectively). The respective values for IgM testing were: IB 36% and 43%; ELISA 42% and 61%. By the end of the follow-up period, IgG and IgM IB gave positive results in 44% and 12%, respectively. In acute and convalescence phase sera, VlsE was the most sensitive protein in the IgG IB (60% and 70% positive, respectively). In the IgM IB, p41i (46% and 57%) and OspC (40% and 55%) were the most sensitive proteins. By the end of follow-up, only the IgM response to p41i was significantly decreased to 24% (vs. 46% before therapy) (p =.036). The immune response to all other proteins did not change significantly over the follow-up.
The IgG IB was more sensitive than the IgG ELISA, whereas the IgM IB was less sensitive than the IgM ELISA. VlsE in the IgG IB and p41i and OspC in the IgM IB were the most sensitive proteins in acute and convalescence phase sera. During long-term follow up, only the immune response to p41i decreased significantly. Thus, the immune response against different Bb antigens is not influenced by antibiotic therapy.

P113

The PDE4 inhibitor Roflumilast shifts the cytokine profile of proinflammatory M-DC8+ blood dendritic cells and reduces their capacity to programm Th1 immune responses

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M-DC8+ blood dendritic cells (DCs) are the largest population of native human blood DCs. They are a major source of the proinflammatory cytokine TNF-α and of the Th1-inducing cytokine IL-12, both of which are abundantly expressed in the chronic phase of the allergic inflammation. In this study, we asked whether the proinflammatory potential of M-DC8+ DCs could be inhibited by a phosphodiesterase (PDE)4-inhibitor. The clinical efficacy of PDE4-specific inhibitors for the treatment of atopic dermatitis, allergic rhinitis and allergic asthma has been documented in several studies.. Furthermore, a beneficial effect of PDE4-inhibitors in the treatment of TH1-dominated chronic obstructive pulmonary disease (COPD) has recently been proven.
RT-PCR analysis revealed that PDE4 is abundantly expressed in M-DC8+ DCs. Within this family (PDE4A-D), a prominent expression of PDE4A, followed by 4B and 4D was found.
The PDE4-inhibitor Roflumilast did not alter the phenotype of M-DC8+ DCs as observed after in vitro maturation. Interestingly, treatment of DCs with Roflumilast for 6 h prior to stimulation with LPS and IFN-γ reduced TNF-α-levels by 60% and IL-12-levels by 90%. Furthermore, a 2-fold increase of IL-10-production was noticed. Maturation of DCs in the presence of Roflumilast did not affect their capacity to promote proliferation of allogeneic CD4+CD45RA+ T cells.
Mature M-DC8+ DCs promote TH1 polarisation. Using Roflumilast-treated DCs for the programming of naïve allogeneic T cells we observed a 30% reduction of IFN-γ producing TH1 cells as assessed by intracellular cytokine staining after restimulation.
These data indicate that PDE4-inhibitors may exert part of their anti-inflammatory activity by inhibition of M-DC8+ blood DCs.

P114

Antibody-based targeting of tumor antigens to dendritic cells in vivo cures mice from B16 melanoma.

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To facilitate antigen presentation dendritic cells (DCs) express receptors that guide antigens through the endosomal pathway, resulting in antigen processing and presentation. The antigen receptor DEC-205 (CD205) is nearly exclusively expressed by DCs and enhances antigen presentation up to 500-fold as compared to pinocytosis. We therefore aimed to use antibodies directed against DEC-205 to load DCs in situ with antigens. A fusion protein of the tumor antigen tyrosinase related protein (TRP) -2 and the green fluorescent protein (EGFP) was coupled to anti-DEC-205 antibodies (αDEC) and injected together with CpGs s.c. into mice. Lymph node cells (LNC) were prepared from draining LN 2h to 24 h later. FACS analysis revealed that 6h after injection of these conjugates up to 20% of the CD11c+ DCs displayed EGFP fluorescence, indicating that EGFP-TRP2-αDEC conjugates had selectively been taken up by LN DCs in situ. To test whether this targeting resulted in induction of TRP2 specific CD8+ T cells, spleen cells from conjugate plus CpG injected mice were restimulated with TRP2-peptide and in ELISPOT assays we were able to detect IFN-γ producing T cells. In contrast no IFNã producers were detected in control samples. Moreover, we demonstrated vigorous induction of TRP2 specific antibodies in TRP2-αDEC-conjugate injected mice, whereas mice that received uncoupled TRP2 protein together with CpGs developed only weak antibody responses. Finally we tested whether injection of the conjugates has a therapeutical effect on developing melanoma. Therefore we seeded B16 melanoma cells s.c. into the hint flank of mice and after the first developing melanoma was visible (diameter approx 1 mm) we injected the mice 2 times in a weekly interval with TRP2-αDEC-conjugates plus CpG and respective controls. Thereafter the size of the developing melanoma was recorded. Here we could show that the growth of the B16 melanoma stopped in nearly 70% of the mice receiving the TRP2-αDEC-conjugates plus CpG, whereas in controls all mice developed severe melanoma. Thus these data show that in vivo targeting of tumor antigens to activated DCs protects against growing melanoma in a therapeutical setting.

P115

Diphenylcyclopropenone treatment of alopecia areata induces apoptosis of perifollicular lymphocytes

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Alopecia areata (AA) is regarded as a T-cell mediated autoimmune disease of the hair follicle. The pathogenesis of AA involves perifollicular CD4+ and CD8+ cells and homing of these T-cells into the skin is mediated by the homing-receptors CD44s and CD44v10. The most effective treatment of AA is the weekly elicitation of a contact dermatitis by application of a contact sensitizer like diphenylcyclopropenone (DCP). The immunological mechanisms underlying this treatment are so far poorly understood. In order to elucidate the cellular mechanisms involved in the treatment of AA with a contact sensitizer, we performed immunohistochemical studies on scalp biopsies of AA-patients before and after DCP-treatment. We compared the expression of CD4, CD8, CD1a, CD68, CD44s, CD44v10, Fas, FasL within the perifollicular infiltrate in untreated patients and in successfully treated patients 1 day and 3 days after application of DCP. Furthermore the number of apoptotic lymphocytes was assessed by TUNEL-staining. Double staining for CD4 and CD25 was performed to identify regulatory T-cells.
Untreated AA showed a perifollicular infiltrate consisting of CD4+ cells, CD8+ cells and CD68+ cells as well as single CD1a+ cells. CD44s was expressed moderately and CD44v10 weakly. 1 day after application of DCP there was a striking increase in perifollicular CD4+ cells and CD68+ cells, the expression of CD44s, CD44v10 and FasL was upregulated and a large number of apoptotic lymphocytes was found in the perifollicular infiltrates. 3 days after DCP-application the number of infiltrating CD4+ cells and CD68+ cells was reduced and showed the same density as in untreated AA but CD8+ cells were almost absent and there was still a large number of apoptotic lymphocytes.
Our data suggest that the elicitation of a contact dermatitis by DCP-application induces apoptosis in perifollicular lymphocytic infiltrates in AA resulting in a removal of CD8+ cells. The apoptosis-inducing cells seem to be part of the early inflammatory infitrate of the contact dermatitis and might be CD4+ regulatory cells.

P116

SCID mice transplanted with human gut as a pre-clinical model for studying the migration of human lymphocytes to the small intestine

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Diseases of the skin frequently occur as complications of inflammatory bowel disease or together with alimentary truct disorders. The recruitment of pathologic T cell populations into the small intestine is mediated by CCL25. This chemokine exclusively interacts with CCR9, which is expressed by essentially all CD4+ and CD8+ T lymphocytes displaying the intestinal homing receptor fÑ4fÒ7. Thus, CCR9 antagonists are potential therapeutics for the treatment of inflammatory bowel diseases (IBD) and may also prevent associated skin disorders.
Studying the in vivo function of human chemokines and their pharmacological inhibition often requires species specific models. To develop a system for proving the efficacy of compounds interfering with the CCL25-CCR9 mediated T cell recruitment in a relevant in vivo environment we established a humanized severe combined immunodeficiency (SCID) mouse model. SCID mice were transplanted with fragments of human fetal small intestine (SCID-hu Gut) and reconstituted with a human T cell line. This line was derived from adult mesenteric lymph nodes and expressed CCR9 and fÑ4fÒ7 integrin. In response to CCL25 this mesenteric lymph node derived T cell line reacted in actin polymerization assay and specifically migrated in the chemotaxis chamber. Homing of human T cells to the human intestine in vivo was induced by intragraft administration of CCL25 and evaluated by flow cytometry and histological analysis using HLA-specific mAbs. CCL25 administration induced a significant migration of CD4+ and CD8+ T cells to the human intestinal graft. The in vivo T cell recruitment was specifically inhibited using a neutralizing mAb against CCL25. This new mechanistic model will allow the preclinical evaluation of human specific compounds interfering with the different steps that regulate homing of T cells to the small intestine and thereby abolishing inflammatory responses in intestinal and related skin diseases.

P117

Is there a coincidence of bullous pemphigoid and psoriasis vulgaris? Results of a clinical and serological study

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An autoimmune pathogenesis for psoriasis is one of several currently favoured concepts while the exact target antigen(s) and mechanisms remain elusive. Humoral or cellular autoimmune phenomena are well-known for other autoimmune disease and may involve the main organ involved as well as other body tissues. Coinciding bullous pemphigoid and psoriasis vulgaris have been casuistically described over the past half decade several times and the pathogenic relation has been mainly attributed to therapeutic procedures which either immunologically alter or make accessible basement membrane antigens and result in autoimmune mediated blistering disease. As a direct relation remains obscure regarding the incidence rates of both diseases, in this study anti-basement membrane antibodies have been examined serologically in a group of patients with psoriasis vulgaris (n=100) and healthy controls (n=50). In addition, serological data have been compared among different therapeutic approaches. Circulating antibodies have been studied on monkey esophagus, human salt split skin as well as in a BP180 ELISA. Only low titered antibodies (< 1:40) have been found among 4.5 % of psoriatic patients studied. No correlation was found to different therapeutic approaches, namely UV-therapy which has in the past been favoured to result in the induction of anti-basement membrane antibodies. Consequently, routine serological testing of psoriatic patients is unappropriate and non-predictive and the described coincidence of psoriasis and bullous pemphigoid seems incidental rather than pathogenically related.

P118

Modulation of the expression of antimicrobial peptides in vivo

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Antimicrobial peptides are a group of small, cationic, cystein-rich peptides with broad-spectrum antimicrobial acticity and were shown to be an important part of the innate immunity of the skin organ. The intention of this study was to monitor the expression of human ©¬-defensins (hBD) -2, -3 and psoriasin (Pso) in human skin grafts on NOD-SCID mice by in situ hybridisation and immuno-histochemical methods.
Healthy human skin was obtained from plastic surgery. Two circular skin grafts of 8 mm diameter were transplanted onto the back of each mouse. Upon grafting after an average of 6 to 8 weeks, stimulatory as well suppressive cytokines like tumor necrosis factor-alpha (TNF-a), interferon-gamma (INF-g) as well as interleukines (IL)-4 and -13 were injected subcutaneously singly or in combination at different dosages in one of the grafts. Phosphate buffered saline served as negative control in the second graft. Expression of antimicrobial peptides was examined using RNA probes generated in-house as well as specific commercial mono- and polyclonal antibodies. Psoriasin was found constitutively expressed in 60% of the skin grafts. Expression was raised after injection of TNF-¥á or INF-¥ã and suppressed upon combined injection with IL-13 as compared to the PBS-control. Similarly, expression of HBD-2 and 3 could be modulated by injection of the different cytokines
In summary, this xenogenic skin transplantation model demonstrates the differential effects of TNF-¥á, INF-¥ã, IL-4 and -13 in the expression of HBD-2, -3 and psoriasin. This might help to understand the pro-inflammatory effects of TNF-¥á and INF-¥ã in several chronic skin diseases like psoriasis and atopic dermatitis.

P119

Protective role of the disruption of the proteinase-activated receptor-2 (PAR-2) gene in the mouse model of endotoxaemia induced by bacteria-derived lipopolysaccharide (LPS)

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Recent findings indicate a role of serine proteases in innate immunity. Proteinase-activated receptor-2 (PAR-2) belongs to a novel subfamily of G protein-coupled receptors with seven trans-membrane domains. It can be activated by serine proteases such as mast cell tryptase, trypsin or leukocyte derived proteinase-3, for example. Some inflammatory mediators such as TNF-α, IL-1α and LPS are known to upregulate PAR-2 expression. However, the simultaneous actions of inflammatory stimuli and PAR-2 agonists are still poorly investigated in both in vivo models and in cultured human cells. In the present study we demonstrated that PAR-2 knockout animals are more resistant to harmful effects of LPS-induced endotoxaemia as compared to wild-type mice. Further experiments with isolated human blood cells, neutrophils and monocytes, also demonstrated that simultaneous stimulation of these cells with PAR-2 agonists and LPS results in enhanced damaging effects as compared to effects after LPS stimulation alone. Among these effects are a) a delayed apoptosis of human neutrophils, which is known to be injuring for surrounding tissues, and b) a strong down-regulation of CD88 (C5a anaphylatoxin receptor) expression on both human neutrophils and monocytes. Interestingly, a decreased expression of CD88 on neutrophils is thought to be associated with an immunosuppressive stage of the immune response in humans during sepsis and HIV. Moreover, a positive correlation between the level of CD88 expression on neutrophils and animal survival during sepsis was recently demonstrated. Therefore, our in vivo and in vitro data clearly suggest that PAR-2 activation may be harmful during endotoxaemia, and such an effect is mediated via immunosuppressive effects on neutrophils and monocytes. Thus, PAR-2 may be involved in the pathophysiology of acute as well as chronic inflammatory diseases by regulating neutrophil and monocyte function.

P120

Construction of a DNA construct allowing the secretion of functionally active chemokines in vitro and in vivo

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CCL21 is a chemokine (CK) expressed in secondary lymphoid organs, exerting chemotactic activity mainly on CCR7+ cell types, including central memory and naïve T cells and mature dendritic cells. Artificial CCL21 expression may thus allow to co-localize different cell types at the site of choice, an important prerequisite for targeted cell migration.
As one can expect the continuous administration of CKs as recombinant proteins at a biologically active dose to be impractical, the goal of this project was to generate a DNA construct allowing the expression and secretion of transgenic CCL21, mediating the functional properties of CCL21 protein in vitro as well as in vivo.
We screened several DNA vectors for expression of their respective transgenic CCL21 and finally constructed a pDNA vector allowing high level expression of CCL21, which was tagged with the reporter sequence V5. Expression of CCL21 was confirmed in transfected COS-7 cells by means of immunocytochemistry and Western blot analysis for CCL21 and V5 tag protein. Secretion of the transgenic protein was determined by CCL21 ELISA in the supernatants of transfected cells. The functional characteristics of expressed CCL21 as a chemotactic agent was confirmed by using transwell migration assays in vitro. In these assays, splenocytes isolated from mice served as responders and supernatants of transfected COS-7 cells as a source of the chemokine. Expressed tagged CCL21 (60 ng/ml) was as efficient as 1 μg/ml of rCCL21 in inducing migration of CCR7+ cells. One possible explanation for the superior effect of transgenic CCL21 may constitute enhanced posttranslational processing more resembling biologically active CCL21. Neutralizing anti-CCL21 antibody was able to block the chemotactic effect of secreted CCL21 thereby further confirming the specificity of the system. In vivo experiments nicely showed ectopic cellular expression of transgenic CCL21 after particle-mediated gene delivery e.g. in keratinocytes and, most importantly, its secretion into the inter-/subcellular space, namely the upper dermis where it efficiently mediates its biological effects. Our studies show that CCL21 can be expressed by genetic means in vitro and in vivo.

P121

IMPROVING THE MIGRATION OF INJECTED, ANTIGEN-CHARGED DENDRITIC CELLS FROM SKIN TO LYMPH NODES. A MOUSE MODEL FOR INCREASED TUMOR VACCINE EFFICIENCY:

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Dendritic cells "loaded" with tumor-specific antigens are increasingly used for adoptive immunotherapy of cancer in clinical trials. The preferred route of administration is via the skin. Few studies have clearly shown, however, that migration of dendritic cells from the injection site to draining lymph nodes is highly inefficient. We have therefore sought to find ways to improve dendritic cell migration in a mouse model that imitates "human" requirements as far as possible. Dye-labeled (CFSE) or congenic (Ly5.1) dendritic cells were injected into the skin of the ear. Numbers and phenotype of dendritic cells arriving in the nodes were evaluated. A co-administration of inflammatory stimuli was less efficient than pretreatment of injection sites with inflammatory stimuli. Such stimuli were pre-injection of cytokines (TNF-alpha) or the epicutaneous application of contact sensitizers (TNCB, picryl chloride) 24h before injecting dendritic cells for vaccination. These pretreatments led to a marked increase of dendritic cells in the lymph nodes and, as a consequence, to an increased antigen-specific proliferation of T-cells. This model will allow to optimize conditions for adoptive transfer of dendritic cells via the skin.

P122

Enhancement of Polymorphonuclear Neutrophil Function by Zinc Histidine

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Polymorphonuclear leukocytes (PMNs) are the primary effector cells of the innate immune system. PMN deficiency results in susceptibility to bacterial infection. We tested whether zinc bound to its physiological ligand, zinc histidine, had an enhancing effect on the respiratory burst of PMNs.
PMNs were isolated from the peripheral blood of healthy donors. Zinc histidine, and for comparison zinc orotate and zinc sulfate (1.05 to 20 mmol/l) were added to PMNs. Opsonized zymosan, serum-opsonized zymosan, zymosan-activated serum, f-met-leu-phe, phorbol myristate acetate and heat-aggregated immunoglobulin served to stimulate PMNs. The respiratory burst was measured by luminol-enhanced chemiluminescence (CL). A solution of 3% hydrogen peroxide served as a cell-free luminogenic system.
We found that both zinc orotate and zinc sulfate virtually completely suppressed the respiratory burst of PMNs with all the different 6 stimuli employed. By contrast, zinc histidine significantly enhanced the respiratory burst of PMN by approximately 100 % ($p<0.01$, Kruskal-Wallis test, Bonferroni correction). In similar fashion, the CL of resting PMNs was also enhanced by zinc histidine. In the cell-free system all zinc compounds quenched CL by 80 - 100%.
Our results demonstrate that zinc-histidine, in contrast to zinc orotate or zinc sulfate, markedly enhanced the respiratory burst of PMNs. It remains to be shown whether this effect can be used therapeutically.

P123

Lack of HLA-class I expression on melanoma cells caused by multiple mutational events

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Cytotoxic T cells have the capability to specifically destroy tumor cells. Different immunotherapeutic treatment strategies are now evaluated in clinical studies for their capacity to mobilize these T cells. On the other hand, tumor cells can escape recognition by cytotoxic T cells via down-regulation or total loss of HLA-class I molecule surface presentation. The molecular nature of these escape mechanisms has to be characterized in detail in order to develop immunotherapies which circumvent tumor defense strategies. Therefore we analyzed the mechanisms contributing to the loss of HLA-class I presentation on four melanoma cell lines.
On none of the analyzed tumor cells surface presentation of HLA-class I molecules could be restored by IFN-γ treatment, leading to the assumption that mutations affecting the beta2-microglubolin gene might be causative for the HLA-class I negative tumor cell phenotype. Indeed, re-expression of HLA-class I molecules was clearly detectable on those cells which could be transciently transfected with a b2m cDNA expression plasmid. To further characterise the genetic defects, total RNA from all cell lines and the positive control cell line Hela was isolated followed by b2m-specific RT-PCR. According to the control, a b2m-specific cDNA fragment was detectable in two of the four cell lines: Sequence analysis of the PCR products revealed that in one cell line a microdeletion of 2 base pairs in exon II of the beta2-microglubolin gene occurred whereas the second melanoma cell line exhibited an insertion of intron sequences between exon I and exon II.
We intended to amplify genomic b2m DNA sequences from those cell lines from which b2m-specific cDNA products could not be obtained, employing different primer pairs for synthesis of overlapping PCR fragments covering the exons and the exon/intron transitions of the b2m gene. In contrast to the control no b2m-genomic DNA sequences could be amplified from these cells, pointing to an extensive deletion of genomic sequences. This could indeed be demonstrated by loss of heterozygosity analysis for several polymorphic microsatellite markers located upstream or downstream of the b2m gene on the long arm of chromosome 15.

P124

Induction of Heme Oxygenase-1 (HO-1) inhibits Dendritic cell Differentiation and Adaptive Immunity

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The strong immunosuppressive potency of the stress protein HO-1 has been proven in several models of autoimmunity and transplantation. The underlying immune mechanisms, however, are poorly characterised. In our study, the potent HO-1 inducer Cobalt-Protoporphyrine IX (Co-PPIX) strongly suppressed T-cell proliferation in mixed lymphocyte reaction (MLR). As possible mechanism we demonstrated a selective Co-PPIX induced increase of HO-1 expression in monocytes associated with depression of accessory molecule expression and stimulatory cytokine secretion. The likewise induction of HO-1 in monocyte-derived dendritic cells (MDDC) by Co-PPIX was associated with an almost complete inhibition of the differentiation, maturation, and function of MDDC. So, a strong decrease of the expression of DC markers (CD1a, CD83) and accessory molecules (HLA-DR, CD86) was observed. Whereas IL-12 secretion was inhibited, IL-10 production increased. The antigen-presenting capacity of Co-PPIX-treated MDDC was strongly diminished in lymphocyte transformation assay and MLR. The specifity of these effects was demonstrated by HO-1 transduction in immature MDDC. Additional investigations regarding effects of Co-PPIX on genome-wide gene expression and signal transduction pathways in monocytes and DC are ongoing. Together these changes indicated a swith of the DCs to an immature, non-stimulatory phenotype. In-vivo, Co-PPIX treatment before challenge dose-dependently deressed ear inflammation in DNFB (Type 1) and TMA (Type 2) induced contact dermatitis in mice. Remarkably, Co-PPIX even more strongly inhibited T-cell-dependent inflammation when applied around sensitisation. We hypothesise that the inhibition of DC differentiation, maturation, and function is a crucial mechanism for the suppression of adaptive immunity by HO-1 induction in-vitro and in-vivo.

P125

In vitro CD8 T cell priming: many antigen presenting cells, or much antigen per presenting cell; that#s the question!

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Naive antigen (Ag) specific T cells can efficiently be activated during in vitro stimulation with professional antigen presenting cells (APCs) involving repeated T-APC interactions. However, not much is known to which extend the number of APCs and the level of presentation per cell (i.e. Ag density) contribute to the expansion of specific T cells and induction of effector functions.
In our experiments we used monocyte-derived dendritic cells (DCs) as professional APCs, and we chose MelanA as a model-Ag, for its well-characterized and highly immunogenic HLA-A2-presented peptide EAAGIGILTV. DCs were transfected with RNA coding for MelanA using electroporation or a lipid-based transfection reagent. These resulted in different percentages of transfected cells and expression levels on a per cell base. In addition different percentages of the DCs were loaded with a MelanA derived analogue peptide (ELAGIGILTV). The DC#s capacity to expand autologous T cells was analyzed by in vitro stimulation and subsequent A2-MelanA tetramer-staining in combination with phenotyping (CCR7/CD45RA) for T cell function. We found that methods that result in higher levels of Ag density per cell are by far superior in generating high numbers of effector CD8+ T cells, even if the Ag was presented only by a small percentage of the DCs, compared to methods that transfect almost 100 % of the cells but yielding lower Ag density. Surprisingly as little as 10% of peptide presenting DCs displayed the same stimulatory capacity as 100% peptide loaded DCs to expand Melan-A specific CD8+ T-cells.
Our in vitro data suggest a prominent role of a high antigen density displayed by few DCs for effective CD8+ T cell activation and thus may have consequences when designing vaccination-strategies with RNA transfected or peptide-loaded DCs.

P126

Making the diagnosis of pemphigoid in a selected cohort of patients by ELISA

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Currently, the serological diagnosis of autoimmune bullous skin disorders mainly relies on the detection of auto-antibodies (auto-Ab) on defined tissue substrates. The goal of this study was to evaluate the diagnostic performance of ELISA assays in the detection of auto-Ab in a cohort of 24 patients with bullous pemphigoid (BP) and 9 patients with mucous membrane pemphigoid (MMP). In these patients, diagnosis of BP and MMP was primarily based on clinical criteria and direct (DIF) and indirect immunofluorescence microscopy (IIF). In 16 of 24 patients clinically diagnosed with BP both DIF and IIF were positive. Eight of 9 patients diagnosed with MMP were positive by IIF, and 6/8 by DIF. Sera from these patients were tested for IgG reactivity with recombinant BP180, BP230 and purified native laminin 5 (LN5) utilizing recently developed novel ELISA assays. A total of 33 sera from BP and MMP patients were first screened for BP180/BP230 reactivity. Additionally, sera from the MMP patients and the BP180/BP230-negative sera were examined by ELISA for LN5 reactivity. The sera of 20/24 patients diagnosed with BP showed IgG reactivity with BP180 and/or BP230 by ELISA. Sera of 3 BP patients who were BP180/BP230-negative were positive in the LN5 ELISA suggesting that the correct diagnosis was rather MMP. Only 1 BP serum, showing linear BM fluorescence in the IIF, was BP180/BP230 and LN5 negative. Sera from 7/9 patients diagnosed with MMP were reactive with LN5, 4/7 patients were reactive both against LN5 and BP180/BP230. One of the two MMP patients who were LN5 negative was reactive with BP180. The second serum was LN 5 and BP180/BP230-negative but reactive by IIF suggesting reactivity to an unrelated antigen. In conclusion, serological diagnosis of BP/MMP by ELISA is at least as sensitive as IIF. Moreover, ELISA analysis with defined autoantigens allows for a more precise characterization of the auto-Ab profile and may be extremely helpful for a better definition of specific subgroups of autoimmune bullous skin disorders with differential clinical prognosis such as BP and MMP.

P127

Dendritic cells use CYTIP to actively regulate T-cell de - attachment

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When T-cells are primed by dendritic cells (DC) to initiate antigen-specific immune responses screening for matching antigen receptor-MHC/peptide pairs takes place in DC-T-cell conjugates. For a successful immune response loosening of the cell-cell interaction either after unsuccessful scanning as well as after successful priming is necessary. While detailed knowledge on molecules involved in the conjugate formation is available, little is known on the mechanisms causing the loosening of these interactions. We have identified the cytosolic protein CYTIP (cytohesin-interactin protein) to be centrally involved in mediating these events. In mature DC CYTIP localizes to the contact zones with T-cells for a short period of time within the first thirty minutes of co-culture. Thereafter, it redistributes within the cytoplasm. Silencing of CYTIP in DC results in stronger adhesion of DC to T cells and to fibronectin. We therefore deduce a role for CYTIP in mediating the rapid detachment of DC from T cells during the early priming events. Thus, CYTIP is the first molecule described to mediate DC-T-cell detachment.

P128

Novel mechanisms of glucocorticoid action on human monocytes

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Glucocorticoids (GC) are still the most widely used immunosupressive agents in clinical medicine. Surprisingly little is known about the mechanisms of GC action on monocytes which play a central role in the pathogenesis of many autoimmune diseases. We therefore analysed the effect of GC treatment on gene expression of human blood monocytes by microarray technology (HG U133 Av2 arrays). The monocytes (>90% purity) were obtained from 4 individual donors and stimulated for 16 h with 10 nM fluticasone propionate. We identified 102 up- and 45 downregulated genes meeting strict statistical criteria (4 independent experiments). Real-Time PCR and flow cytometry confirmed high reliability of array data.
To get accurate statistical information about overrepresented groups of functional related genes the percentage of genes belonging to a distinct functional group for the regulated and for all genes has to be statistically compared. We used the GENMAPP software (www.genMAPP.org) and information present in the Entrez Gene database (Gene references into function) for these calculations. Among expected results (e.g. downregulation of genes represented by the gene ontology identifier "immune response" (www.geneontology.org)) we also identified biological processes which were affected in an unexpected way by GC treatment. Genes related to "phagocytosis" and "cell motility" were upregulated while genes related to "cell adhesion" and "apoptosis" were downregulated. In order to confirm these biostatistical results we analysed monocyte functions after GC treatment in vitro. As predicted adherens of GC treated monocytes to plastic surfaces was decreased. GC treated monocytes showed a smaller rate of spontaneous apoptosis and were even protected from staurosporine induced cell death. However they showed increased chemotaxis to fMLP and increased phagocytosis of leishmania major and latex beads.
In conclusion GC treatment did not result in a global inactivation of monocytes neither on the gene expression nor on the functional tier. GCs rather activate distinct monocyte functions which may be important for controling the immune response. These results support the concept of an alternative activated antiinflammatory monocyte/macrophage subtype induced by GC treatment.

P129

Eliminating circulating desmoglein-reactive autoantibodies in pemphigus patients by immunoadsorption (IA)

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Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) represent autoimmune disorders of the skin and the mucous membranes, mediated by autoantibodies directed against the extracellular domains of desmoglein 3 (Dsg3) in PV and desmoglein 1 (Dsg1) in PF, respectively. The therapy is based on glucocorticoids and steroid-sparing immunosuppressive drugs. In this study we investigated the efficacy of IA as an adjuvant treatment in addition to the immunosuppressive therapy in a group of four PV- and two PF-patients. IA was performed using Globaffin® adsorber columns on four consecutive days representing one treatment cycle. Each IA-cycle was followed by a four week interval. One PV-patient underwent four treatment cycles, three PV-patients received two cycles and the two PF-patients were treated with one IA-cycle. Depending on the severity of the pemphigus lesions two PV- and one PF-patient received i.v. cyclophosphamide treatment on day four of each IA-cycle and once a month for a total of six doses. Anti-Dsg1/Dsg3-IgG autoantibodies were measured by ELISA. Serum samples were taken daily before and after each IA-treatment and afterwards once a week during the follow-up period. All six pemphigus patients showed an overall significant reduction of Dsg-reactive autoantibodies. The ELISA score was determined as pemphigus index value (PIV) representing the titres of autoantibodies in the serum . In all pemphigus patients PIV dropped by 5-16 fold during IA-treatment (PIV values dropped by 9,5 $\pm$5,2 fold after the initial IA-cycle). Noteworthy, there was a rise of PIV values between each IA-treatment which might be due to redistribution of tissue-bound autoantibodies into the circulation. Comparing PIV after the first IA-cycle with day 14 after the first IA, PIV scores rose by 1,0-3,7 fold (2,0 $\pm$1,1). However, except for one PV-patient, autoantibody titres remained on a significantly lower level after IA-treatment compared to the initial values over a period of up to nine months. These serological findings were accompanied by rapid clinical improvement of cutaneous and mucosal pemphigus lesions. IA was safe and well tolerated without any severe side-effects.

P130

In vivo-remission spectroscopy measurements on microcirculation of chronic venous leg ulcers treated with PROMOGRAN* matrix

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Background:Chronic venous leg ulcers are characterized by delayed healing, prolonged inflammation and impaired microcirculation. Topical therapy of leg ulcers with a collagen/oxidised regenerated cellulose-based matrix e.g. PROMOGRAN*Matrix has been shown to rebalance the chronic wound microenviroment by normalizing the MMP/-TIMP ratio in wound exudates. PROMOGRAN* Matrix therapy exerts a positive effect upon granulation, but wheather it has an effect on vascularisation and microcirculation has not been yet eluicated. Patients and methods: The present observational study included a total of 40 patients with venous or mixed venous-arterial chronic leg ulcers (25 females, 15 males, aged 43-93).30 patients (19 f, 11 m) were treated with PROMOGRAN*Matrix, 10 control patients (6 f, 4 m) were treated with standard therapy. Clinical assessment of quality of granulation tissue of wounds-was protocoled using a standardized protocol document. In addition measurements on wound status, especially microcirculation of granulation tissue, were performed using digital photography and contact-free remission spectroscopy with the SKINREM device. Measurements were performed before and after 1 and 2 weeks of PROMOGRAN* Matrix therapy and at ten previously defind measuring points along the wound bed and the surrounding skin. Results: Up to now not all results have been evaluated, but statistical analysis on results of 17 patients have been completed. In summary improved granulation in 14/17 patients, decrease of ulcers size in 12/17 patients, reduction of discharge and inflammation in 14/17, and reduction of pain in 14/17 patients was observed. There were no PROMOGRAN* Matrix therapy related adverse effects observed. The therapy was well tolerated and the product handling was easy. In PROMOGRAN* Matrix therapy responders showed an increased HbO2 supply (i.e. improved microcirculation) of the wound bed after one week of treatment. Results on remission spectroscopy measurements showed edema reduction and improved wound healing. These results were associated with an increased microcirculation in the surrounding skin.Conclusion: This is the first evidence of improved microcirculation in chronic leg ulcers induced by PROMOGRAN* therapy in both the wound bed and the surrounding skin. These findings contribute to the basic understanding of the mode of action of PROMOGRAN* therapy.

P131

Intralesional Application of Phospatidylcholin for the Reduction of Lipomas: Preliminary Results

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Phosphatidylcholine, a lecithine extracted from soybeans is able to lower serum cholesterol to a certain extent. Intravenous application may prevent fat embolism in polytraumatic patients. In certain wellness and beauty clinics all over the world it is now applied intralesionally for the reduction of undesired fat depots in cosmetically disturbing body areas, disregarding the fact that clinical studies for the scientific background of this treatment are insufficient.
To prove the ability of phosphatidylcholin (Lipostabil N®) for the reduction of fat we designed an ethics committee approved pilotstudy for the minmal invasive reduction of lipomas by intralesional application of this substance. Lipomas are fequently found benign tumors of subcutaneous fat tissue, their removal may be desired because of functional or aesthetical disturbance. So far this required surgical excision or liposuction.
In twelve patients with lipomas phosphatidylcholin was injected intralesionally three times in a three-week interval. The size of the lesions was measured sonografically before the first treatment and 6 weeks after the third. Reduction of volume (median minus 36,43%) could be achieved in 66% of the lesions. One third of the lipomas turned out to be larger at the end of the study than before the treatment (median plus 20,99%).
Acceptable reduction of lipoma size after intralesional application of phosphatidylcholin (Lipostabil N®) could be achieved only in a few cases whereas some of the lipomas enlarged. The fact that some of the lesions increased in size and the side effect that all lesions revealed some extent of fibrosis showing a higher sonografical density in the follow up sonografy need further investigations.
Contemplation on the off-label intralesional use of phosphatidylcholin (Lipostabil N®) as a "fat burner" for the reduction of aesthetically disturbing fat depots is advisable.

P132

Microenviroment modulation of chronic wounds influenced by the collagen wound dressing Suprasorb C® - effect on platelet-derived growth factor and coagulation factor XIII

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Introduction: Non-healing wounds are lacking of essential growth factors e.g. platelet-derived growth factor (PDGF) and the coagulation Factor XIII. This is due to increased proteolytic degradation by proteases as neutrophile elastase. In order to support the normal wound healing process the protection of growth factors is required. Within the presented study we investigated the ability of Suprasorb® C, a special wound dressing composed of collagen type I, to protect PDGF BB and Factor XIII from proteolytic degradation.
Material and methods: Suprasorb C® was cut to pieces by means of punch biopsies. These pieces were incubated in the presence or absence of wound fluid with PDGF BB or Factor XIII solutions. Subsequently, the supernatants were collected and the concentration of residual PDGF BB could be determined by means of ELISA (R&D). For Factor XIII quantification we used the Berichrom Assay (Dade Behring). Samples without Suprasorb C® and with another wound dressing (Promogran, Johnson & Johnson, U.K.) were used as controls. Additionally, wound dressings were washed twice after incubation to recover bound PDGF BB and Factor XIII.
Results: A significant ($p<0,001$) decrease of PDGF BB concentration could be found in samples with Suprasorb C® and Promogran compared to control samples after 1 hour of incubation. In the presence of wound fluid already after 30 minutes a significant lower concentration of PDGF BB was observed. Bound growth factor could be recovered to an extent of about 20 percent. Similar results were obtained in analogous experiments with Factor XIII. However, the decrease of the concentration of unbound Factor XIII was not significant.
Conclusions: These data confirm the ability of Suprasorb C® and Promogran to protect the platelet-derived growth factor BB from degradation. In comparison, only an insignificant protective effect on the coagulation factor XIII could be found.

P133

Microenviroment modulation of chronic wounds influenced by the collagen wound dressing Suprasorb C®

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Introduction : A lot of studies have shown, that exudates from non-healing wounds contain elevated levels of proteolytic enzymes, like matrix metalloproteinases (MMP) and neutrophile elastase. The levels of inflammatory immune modulators, e.g. cytokines, are also increased significantly. Therefore the reduction of these proteolytic enzymes and pro-inflammatory proteins seems to be a suitable way to support the normal wound healing process. The aim of this study was the investigation of Suprasorb®, a special wound dressing consisting of bovine collagen. The ability of this material to bind neutrophile elastase from a defined elastase solution as well as from wound fluid samples should be tested.
Material and methods : Suprasorb® was cut to pieces of different sizes by means of punch biopsies. These pieces were incubated up to 24 hours with 1 mL of elastase solution or wound fluid, respectively. Subsequent the supernatants were collected and the concentration of unbound neutrophile elastase could be determined by means of an ELISA (Milenia). Samples without Suprasorb® and with bacterial cellulose were used as controls.
Results : The decrease of neutrophile elastase concentrations depending on the incubation time and the size of the Suprasorb® pieces could be found. The reduction of elastase is significant ($p<0,05$) after 24 hours for 0.2 cm2 pieces of Suprasorb® and already after 4 hours for 0.5 cm2 ($p<0,001$) sized pieces in a defined enzyme solution. Neither in control samples without Suprasorb® nor in samples with bacterial cellulose instead of Suprasorb® a decrease of unbound elastase was observed. Suprasorb® can also reduce the concentration of neutrophile elastase in wound fluid samples. 0.5 cm2 sized pieces of Suprasorb® caused significant lower concentrations of neutrophile elastase in 1mL wound fluids obtained from a patient suffering from venous insufficiency and peripheral occlusive disease.
Conclusions : The results of this study demonstrate the ability of Suprasorb® to reduce the concentration of neutrophile elastase in a dose dependent manner in enzyme solutions as well as in wound fluids.The data confirm that Suprasorb® can absorb fluids very efficiently because of its porous structure and its capillary activity.

P134

Oxidative stress results in selective oxidative damage and activation of macrophages with enhanced NO and elastase release. Possible implications for the hostile microenvironment in chronic venous leg ulcers

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Chronic venous leg ulcers fail to progress through the normal pattern of wound repair involving inflammation, granulation tissue formation and remodelling, but instead remain in a chronic inflammatory state with little signs of healing. Reactive oxygen (ROS) and nitrogen species (RNS) produced by phagocytes, and iron originating from blood cell hemolysis have been implicated in the impaired healing of chronic venous ulcers, but little direct evidence is available. Here we analyze the extent and the functional implications of oxidative damage in cells infiltrating chronic ulcers. Immunohistological analysis of 8 skin biopsies from patients with chronic venous leg ulcers showed a prevalence of macrophages (CD68+) in the lesional dermis, while in 5 acute wounds they were almost absent at day 5 and granulocytes (CD66+) predominated in the first two days after wounding. Deleterious oxidative stress was evidenced by staining for the oxidative damage marker 8-hydroxy-2-deoxyguanosine (8-OHdG), and for nitrotyrosine (NT), an end product of protein nitration. In chronic, but not acute wounds, we could detect a strong, selective immunoreactivity for 8-OHdG and NT in macrophages, and the accumulation of iron. In vitro, mouse macrophages treated with oxidative stress-inducing H2O2 stained positive for 8-OHdG and NT and functionally produced in an iron-dependent manner($p<0.05$) significantly more NO ($p<0.003$) and elastase ($p<0.02$) than control cells .
Our observations confirm for the first time in vivo the and persistence of selective oxidative stress conditions in macrophages in chronic wounds which is driven by an iron-dependent Fenton reaction. The enhanced release of NO, may be directly cytotoxic by inhibiting critical mitochondrial enzymes, or may combine with ROS to form detrimental peroxynitrite with nitrosative protein, lipid and nucleic acid damage, as well as the enhanced release of elastase, leading to degradation of extracellular matrix proteins and growth factors. In the microenvironment of chronic wounds these factors are likely to further amplify the hostile environment and impaired healing. We suggest that future therapeutic strategies aimed at reducing oxidative stress may improve healing rates in chronic leg ulcers.

P135

The aggravating role of staphylococcal toxins in psoriasis

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The aggravating role of S. aureus superantigens is well known in atopic dermatitis (AD) but has not yet been proven in psoriasis. We investigated the distribution of S. aureus in the skin and nares of patients with AD, psoriasis vulgaris (PS), erythroderma, skin infections, sepsis, and in healthy controls. A SET-RPLAR latex agglutination test was performed to determine staphylococcal enterotoxins A,B,C, and D.
S. aureus was cultivated from lesional skin of 22/25 patients with AD and 15/25 patients with PS. Isolated strains were toxigenic in 44% in AD and in 36% in PS patients. The activity of disease in AD and PS according to the SCORAD or PASI score, respectively, correlated significantly ($p=0.001$) with an isolated toxigenic strain in both diseases. S. aureus from skin infections was toxigenic in half of the patients. All patients with erythroderma harboured S.aureus, mostly on their skin. In AD, sepsis and skin infections, toxin C and in PS toxin B was most often detected. S. aureus was cultured in 12% of healthy persons. These strains were toxin negative.
In our study, S. aureus was present in more than 50% in patients with AD and PS. We found that the severity of AD and PS significantly correlated to enterotoxin production of the isolated S. aureus strain. On the basis of experience with AD, antibiotic treatment could also be an important supporting regimen in managing PS.

P136

Leflunomide inhibits eotaxin but not eotaxin-3 release by human fibroblasts

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Leflunomide is a novel immunomodulatory drug applied in rheumatoid arthritis as well as psoriasis arthritis. Leflunomid's active main metabolite A 77 1726 blocks dihydro-orotate dehydrogenase, the key enzyme of pyrimidine-de novo-synthesis. In murine cells, A 77 1726 has also been demonstrated to interfere with signal transduction through NFκB. As leflunomide has been successfully applied in diseases characterised by eosinophil skin infiltration, such as atopic dermatitis and bullous pemphigoid, we studied its influence on eotaxin release and expression.

Human dermal fibroblasts were stimulated with TNF-α and IL-4. Leflunomide or A 77 1726 were added at varying concentrations. Supernatants were analysed for eotaxin and eotaxin-3 by ELISA (R&D Systems, Minneapolis,USA). In other experiments, RNA was extracted and analysed for eotaxin mRNA by hybridization with specific probes and colorimetric quantification (QuantikineR, R&D Systems, Minneapolis, USA).

Stimulation of fibroblasts with IL-4 with and without TNF-α induced release of eotaxin-3 (appr. 1000 pg/ml). IL-4 and TNF-α synergistically induced eotaxin(appr. 10000 pg/ml). In contrast to the inactive prodrug leflunomide, A 77 1726 induced a 98% suppression of eotaxin release. This was accompanied by a strong suppression of eotaxin expression. Eotaxin-3 release was not affected by A 77 1726.

Leflunomide's active metabolite inhibits eotaxin expression and release in vitro at concentrations meaningful in vivo. As eotaxin as well as eotaxin-3 expression depend on UTP-levels sufficient for mRNA-synthesis, the mechanism of action is probably not due to inhibition of pyrimidine-de novo-synthesis. A 77 1726 has been demonstrated to inhibit IκB-degradation, necessary for TNF α-induced signalling through NFκB. Eotaxin-3 expression seems to depend solely on STAT-6, rendering it resistant to leflunomide's action. The in vivo consequences of leflunomide's differential action on eotaxins remain to be studied.

P137

Anti-L-selectin therapy is not effective in psoriasis: a randomized trial

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The majority of circulating T-, B-, NK cells, monocytes, granulocytes and other immune cells express L-selectin on their surfaces. L-selectin is an adhesion molecule involved in leukocyte interactions with vascular endothelial cells. This process plays an important role in initiating and maintaining inflammation in various diseases. The blockade of L-selectin and thus the inhibition of tissue infiltration of leukocytes, dendritic cells and macrophages is an attractive option for targeted therapy of inflammatory diseases.
We performed a multi-center, randomized, placebo-controlled trial to investigate the efficacy and safety of a recombinant humanized monoclonal antibody to L-selectin, BNP001 (Aselizumab), in the treatment of psoriasis.
21 patients with moderate/severe psoriasis were selected for this study. 14 patients received 1 infusion/week of BNP001 for 4 weeks. To 7 subjects placebo infusions were given. The treatment was well tolerated. After 4 weeks treatment and 6 weeks follow up observation there was no significant reduction in PASI in the BNP001-treated group compared to the placebo group. Immunohistochemical analysis of biopsies taken from psoriatic skin lesions showed no significant reduction in numbers of CD3+, CD4+ and CD8+ lymphocytes in the dermis or epidermis. However, a decrease of L-selectin (CD62L) staining in lesional skin was observed (29.3 cells/mm2 before and 12.3 cells/mm2 after therapy). A marked decline of CD62L staining was also detected on granulocytes in BNP001-treated patients by flow cytometry. There were no significant changes in the peripheral blood NK cell and CD3+, CD4+ or CD8+ cell counts. This data show that anti-L-selectin treatment is well tolerated but not clinically effective in the treatment of chronic plaque psoriasis.

P138

Demonstration of antipsoriatic efficacy of a new topical formulation of a small molecule selectin antagonist bimosiamose

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The attraction of leukocytes to tissues is essential for inflammation and the host response to infection. Psoriasis vulgaris is a T-cell mediated inflammatory skin disease with a prevalence of 2-3 % in the general population. 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. In T-cell-mediated diseases this process is a crucial event in initiating and maintaining inflammation. Therefore, selectins are an attractive target for the development of new anti-inflammatory therapeutics. We investigated topical bimosiamose, a synthetic pan-selectin antagonist in 12 subjects with stable psoriatic plaques in a randomised two-center vehicle controlled study, double blind for the study preparations and observer-blind for the comparators. Measurements (sonography, chromametry, photodocumentation and clinical assessments) were made at baseline (day 1) and on days 8 and 12. Analyzed parameters of the employed psoriatic plaque test were erythema intensity (mean redness) measured by chromametry and infiltrate thickness of the plaque as measured by 20 MHz sonography. Mean redness values remained nearly constant over time for bimosiamose microemulsion and its corresponding vehicle. Thickness of the plaque infiltrate did not change after 10 days of treatment in the vehicle group. In contrast, bimosiamose microemulsion treated plaques demonstrated a significant decline in infiltrate thickness after 10 days of treatment as compared to its corresponding vehicle (-10.5%, p=0.019) and as compared to its own baseline (-13%, p=0.0007). In this phase IIa exploratory trial, a new topical formulation of the small molecule selectin antagonist bimosiamose has been investigated. These data suggest that topical selectin antagonists may be effective in the treatment of psoriasis.

P139

The binding capacity of the collagen wound dressing Suprasorb® C for inflammatory cytokines

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Introduction: Chronic wounds are an important and persistent problem in dermatology. Non-healing chronic wounds, e.g. diabetic or venous ulcers, contain significantly higher concentrations of inflammatory cytokines as IL-1β, IL-6 and TNFα compared to acute wounds. Chronic wounds persist in the inflammatory phase of the normal healing process and often remain non-healing for month or even years. Therefore the reduction of these inflammatory cytokines in the wound fluid seems to be a suitable way to stop the vicious circle of inflammation and diminished epithelization and thus to support the normal wound healing process.
Material and methods: Within the presented study the ability of Suprasorb® C, a wound dressing consisting of bovine collagen type I (Lohmann & Rauscher), to bind IL-1β, IL-6 and IL-8 was investigated. The wound dressing was cut to pieces and incubated in vitro with cytokine solution or wound fluid. Subsequently, the supernatants were collected and the concentration of unbound cytokines was determined using enzyme-linked immuno assays (Milenia, Germany).
Results: As the presented data show, Suprasorb® C is able to bind cytokines from a definite solution as well as from wound fluid. The concentration of unbound IL-1β and IL-6 decreases with prolonged incubation time. Significant less IL-1β was quantified in the supernatant already after 1 h of incubation. After 24 h the concentration of unbound IL-6 was significantly decreased. In contrast, the collagen wound dressing showed no capacity to bind IL-8.
Conclusion: The results of this study demonstrate the ability of Suprasorb® C to bind the inflammatory cytokines IL-1β and IL-6 efficiently.

P140

Anti-CD 20 Monoclonal Antibody (Rituximab) in the Treatment of Pemphigus

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The treatment of severe, protentially life-threatening autoimmune blistering disorders such as pemphigus is still challenging and therapeutic options in patients with recalcitrant disease are often limited. To deplete disease causing B cells that produce autoantibodies, we treated five pemphigus patients with the monoclonal anti-CD 20 antibody rituximab. Rituximab was administered intravenously at a dosage of 375 mg/m2 once weekly for four weeks. The significant clinical improvement in all patients after rituximab was accompanied by a marked reduction of the immunosuppressive co-medication. The mucocutaneous lesions gradually improved in all patients and complete healing was observed in 2 patients. Rituximab treatment was well tolerated, adverse effects such as nausea, vomiting, facial edema, chills or cough occurred mostly during the first intravenous administration and were controlled by paracetamol and antihistamines. Depletion of CD20 lymphocytes occurred rapidly after the last infusion of rituximab and the values returned to normal after 6-15 months. Interestingly, the CD20 count in one patient is still below normal 3 years after the last application of rituximab. The variability of the persistence of B cell depletion is not clear, recent work in patients with systemic lupus erythematodes suggests a correlation between polymorphisms of Fc receptors on effector cells and the degree of B cell depletion and response to rituximab. Our observation that despite clinical improvement the antibody titre to desmoglein 1 and 3 remained constant in two patients is in line with the assumption that plasma cells with longer life spans exist. In conclusion, this study documents the favorable effect of a single course of rituximab in pemphigus. The therapy was effective in cases refractory to standard immunosuppressive agents such as azathioprine, methotrexate and mycophenolate mofetil and was long-lasting.

P141

Influence of cyclodextrins and their derivatives on the generation of ROS in HaCaT keratinocytes and leukocytes

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Introduction: Cyclodextrins (CDs) are cyclic oligosaccharides bound by α-(1,4) glycosidic bonds. α-CD are comprised of 6, β-CD of 7 and γ-CD of 8 α-D(+)-glucopyranose units. CDs form stable rings with a hydrophobic interior which allows the formation of inclusion complexes with a variety of chemical substances stabilized by hydrogen bonds and hydrophobic forces. In this way a lot of organic compounds can be included. Such kind of ligand-receptor complexes can be used for different applications, e.g. for a transdermal therapeutic system (TTS). CDs can increase the solubility and availability of drugs, but there is only a little known about their effects on the generation of reactive oxygen species (ROS). Here we provide evidence that CDs and chemically modified derivatives can modulate the concentration of reactive oxygen species (ROS) in a dose-dependant manner.
Material and Methods: CDs were purchased from the Wacker Chemie GmbH (Munich, Germany). Samples were dissolved in deionised water to 0.1 % (w/v), 0.5 % (w/v), 1 % (w/v) and 2 % (w/v). The ability to scavenge ROS was assessed using the chemiluminescent ABELÒ Antioxidant Test Kit specific for superoxide and other free radicals containing PholasinÒ (Knight Scientific Limited, Plymouth, UK). The ROS formation from human leukocytes were determined using a lucigenin-based chemiluminescence assay. In HaCaT cells, ROS were measured by the formation of the fluorescent dye 2#,7#-dichloro fluorescein.
Results: Natural CDs have shown a slight dose-dependent anti-oxidative effect whereas methylated and hydroxy propylated CDs show considerable pro-oxidative behaviour.
Conclusion: CDs and their derivatives were widely used in the pharmaceutical field. As the presented data confirm the anti-oxidative capability of natural CDs and the significant pro-oxidative effect of modified CDs should be taken into account for in vivo applications.

P142

Effects of a chemical sunscreen on UV induced changes of different histological features in melanocytic nevi

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Exposure to UV radiation can lead to clinical, histological and ultrastructural changes in melanocytic nevi. In this study we investigated the effect of a sunscreen on the UV radiation-induced changes of different histological features in melanocytic nevi.
Twenty-six melanocytic nevi were exposed to three MEDs of solar simulated UV radiation. One half of each nevus was treated with a sunscreen (SPF 11) containing an UVB and UVA filter 20 minutes before irradiation. The other half was unprotected during UV irradiation. The nevi were excised 7 days after UV irradiation. For each biopsy, a single section was reviewed by one of the authors to record the following criteria: thickness of the epidermis, pigmentation of the basal layer, dilated vessels in the papillary dermis, dilated vessels in the reticular dermis, melanocytes in the upper epidermis, aytpical melanocytes in all epidermal layers, dentritic melanocytes in the epidermis, and sun burn cells. Additionally sections immunohistological stained with LCA, S100, MIB1 and HMB-45 antibodies were analysed.
Comparing the sunscreen treated halves of the nevus with the unprotected halves of the same nevus only a significant stronger staining with HMB-45 was found (Wilcoxon signed rank test $p<0.02$). Furthermore in 16 nevi the unprotected half showed more atypical melanocytes in all epidermal layers but these differences were not significant ($p<0.06$).
The sunscreen gave protection against UV radiation induced activation of melanocytes in melanocytic nevi and reduced the formation of atypical melanocytes.

P143

Treatment of Psoriasis with the Recombinant Human Tumor Necrosis Factor Alpha Receptor Fusion Protein, Etanercept

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Psoriasis has shown improvement during anti-TNF-alpha treatment. We compared the clinical and histological effects of TNF blockade with etanercept monotherapy on psoriatic skin lesions.
Eight patients aged 31 to 58 years, with psoriatic arthritis suffering from plaque-type psoriasis were treated. Patients received etanercept, 25 mg, subcutaneously twice weekly for 24 weeks. Only skin emollients were allowed as concomitant treatment. Efficacy for the treatment of skin lesions was evaluated using the Psoriasis Area and Severity Index (PASI). Histological sections were prepared from biopsy specimens of uninvolved and lesional skin at week 0 and from lesional skin at week 12 to measure epidermal thickness (acanthosis).
We found that the PASI diminished from 19.7 ± 3.0 at week 0 (100%) to 8.8 ± 2.2 at week 12 (43%) and 5.9 ± 1.6 at week 24 (29%) (Mean $\pm$ SE). Epidermal thickness from lesional psoriatic skin diminished from 0.40 ± 0.04 mm at week 0 to 0.26 ± 0.04 mm at week 12; uninvolved epidermis measured 0.13 ± 0.02 mm. No adverse effects were noted.
We conclude that in patients with psoriatic arthritis, etanercept monotherapy over 24 weeks markedly improves psoriatic skin lesions as can be verified by pathohistological skin sections.

P144

The soluble variant of the vascular endothelial growth factor receptor VEGFR-1: a potential mediator in wound healing

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The endothelial cell-specific mitogen vascular endothelial growth factor-A (VEGF-A) is a potent regulator of angiogenesis during wound healing. The soluble form of the VEGF receptor VEGFR-1 (sVEGFR-1) is a strong and specific inhibitor of VEGF-A signaling and has been characterized as a potent inhibitor of angiogenesis. We investigated the hypothesis whether sVEGFR-1 plays a role during tissue repair and evaluated the expression of sVEGFR-1 in healing and non-healing human wounds. RT-PCR analysis indicates that the full length receptor VEGFR-1 and its splice variant sVEGFR-1 are expressed in normal skin and non-healing wounds. ELISA and western blot analysis demonstrate that sVEGFR-1 is released at low levels in wound fluid obtained from normal healing wounds averaging 2.2 +/- 2.0 ng/ml (n=11), and at significant higher levels in chronic non-healing wounds with a mean sVEGFR-1 concentration of 9.3 +/- 3.2 ng/ml (n=16) ($p<0.001$). Only in those chronic wounds, which eventually entered a phase of granulation tissue formation and finally wound closure, wound healing progression correlated significantly with a decline in sVEGFR-1 levels ($r=0.92$; $p<0.0005$). Northern analysis of cultured HUVE cells exposed to wound fluid indicate, that wound fluid obtained from chronic non-healing wounds contains mediators that are able to enhance the expression of sVEGFR-1 mRNA. This report suggests that sVEGFR-1 represents a regulatory molecule during wound repair. Our findings lead to the intriguing hypothesis as to whether the sVEGFR-1 level detected in wound fluid can be of predictive value for differentiating healing and non-healing wounds. An indicator for healing would be of great value to assess disease severity and progression of the chronic wound, and might serve as predictive indicator for the efficacy of certain therapy regime.

P145

The expression of leukocyte-chemoattractive factors suggest a pathophysiological link between psoriatic skin and bone disease

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Psoriasis is a chronic inflammatory disorder characterized by sterile infiltration of skin lesions with inflammatory cells, primarily T cells and neutrophils. Up to 35% of patients also suffer from concomitant joint disease. The similarities between skin and bone affection are particularly prominent in a variant of psoriatic arthritis called chronic recurrent multifocal osteomyelitis (CRMO). CRMO is typically associated with pustular psoriasis and presents as a chronic relapsing primary aseptic osteomyelitis characterized by infiltration of the metaphyses of tubular long bones, clavicles, or vertebrae with neutrophils and lymphocytes. The factors involved in the influx of inflammatory cells in CRMO have not yet been clearly identified.
Immunohistochemical and in situ hybridization studies of bone specimen obtained from a patient with CRMO revealed the expression of several leukocyte-chemoattractive factors including interleukin-8, interleukin (IL)-16 and lymphotactin (Ltn). While the expression of IL-16 and Ltn was largely confined to infiltrating T cells, osteoblasts were also identified as producers of chemotactic factors including IL-8 and MIP-3α. To further investigate the posssible role of osteoblasts as sources of chemokines in CRMO, primary osteoblast cultures (n=5) were treated with TNF-α and IL-1β to mimick the proinflammatory environment present in psoriatic arthritis. Investigation by cDNA array and quantitative real-time RT-PCR techniques showed a strong induction of several chemokines, among them IL-8, MCP-1, MCP-2, MIP-3α, IP-10 and RANTES. The cytokine-induced production of these factors was confirmed on the protein level by ELISA, and could not be suppressed by co-incubation of osteoblasts with cyclosporin, a drug used in the treatment of CRMO. Our study provides evidence for a role of leukocyte-chemoattractive factors in the pathogenesis of CRMO. Under the influence of pro-inflammatory cytokines, osteoblasts, in a certain analogy to the role of keratinocytes in psoriatic skin disease, may acquire non-professional immune functions and contribute to the formation of bone lesions in psoriasis.

P146

SILVER-LOADED CELLULOSIC FIBERS WITH ANTI-FUNGAL AND ANTI-BACTERIAL PROPERTIES - NEW BIO-ACTIVE FIBERS FOR INTELLIGENT TEXTILES: SEACELL® ACTIVE

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The skin is the interface between the body and the sometimes harsh environment while textiles are the tissues with the longest contact to human skin. The increasing demand for "intelligent" and "bio-active" textiles resulted in the development of a new fiber called SeaCell® Active. The natural, cellulose and seaweed based SeaCell® fibers served as a functional carrier for the active compound silver, which is known for more than one century to exert antifungal and antibacterial activity. The seaweed-based, silver loaded Lyocell fiber SeaCell® Active contains the minerals calcium, magnesium and sodium, which are known to play a key role in skin homeostasis.
The present study revealed an excellent antifungal activity against different fungi from the Candida family (Candida albicans, Candida parapsilosis, Candida glabrata, Candida tropicalis and Candida krusei). Candida albicans, is responsible for widely encountered itching skin infection with warm, moist and occlusive conditions e.g. under the armpits, under the breasts as well as in the genital and anal regions. Other Candida species like C. parapsilosis (onychomycosis), C. glabrata (genital infection), C. tropicalis and C. krusei were also susceptible against the SeaCell® Active fibers. This effect was quantified in a Neubauer cellcounting chamber. The most striking result was the dose-dependant manner (percentage of SeaCell® Active fibers) of the antifungal activity.
In a second test series we could show activity of the silver-covered seaweed-based fiber against the growth of the bacteria strains Staphylococcus aureus and Escherichia coli in a dose dependant manner.
In summary these results demonstrate the antifungal and antibacterial effect of SeaCell® Activ. This fiber seams to be suited for bio-active textiles in specific body regions and skin conditions with a susceptibility for fungal and bacterial infections namely with Candida species, Staphylococcus aureus and Escherichia coli.

P147

Psoriasin (S100A7) is secreted in E. coli-cidal concentrations on human skin in vivo.

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Human healthy skin is continuously exposed to bacteria, but is surprisingly resistant to the common gut bacterium E. coli. Recently we identified the S100 protein psoriasin as E. coli killing compound from healthy human stratum corneum extracts. We could demonstrate that psoriasin-mRNA is inducible in primary keratinocytes by proinflammatory cytokines and contact with bacteria. In vivo treatment of human skin with neutralizing anti-psoriasin antibodies inhibited its E. coli killing properties suggesting that psoriasin functions as the major E. coli-cidal factor.
Using a newly developed sandwich-ELISA we could show that psoriasin secretion is upregulated in keratinocytes in vitro after stimulation with proinflammatory cytokines and bacterial culture supernatants. To get detailed information about psoriasin secretion in vivo washing fluids (10 mM sodiumphosphate buffer, pH 7.4) derived from 17 different body sites were collected from 8 healthy volunteers. Significant bactericidal levels of psoriasin were found on the skin surface in all donors depending on the location (median secretion: 5,1 to 46,2 ng/cm²). To determine whether psoriasin can also be recovered from lipid extracts additional experiments were performed using acetone for washing. In agreement with psoriasin immunoreactivity and the hydrophobic properties of the protein we found high amounts of psoriasin in lipid-rich skin regions (e.g. scalp skin) as compared to forearm acetone extracts. Interestingly, experimental application of E. coli-culture supernatants on the forearm skin under occlusion resulted in a significant increase of psoriasin secretion.
In summary, this study demonstrates that psoriasin is induced in keratinocytes in vitro and in vivo. The site-specific secretion of psoriasin observed in vivo may derive from local microbial induction. Detection of significant bactericidal amounts of psoriasin on the skin surface could explain for the first time the unexpected resistance of human skin towards E. coli.

P148

Chimeric papillomavirus-like particles (VLP) that display HPV16-L2 peptides induce neutralizing antibodies to genital HPV 11 and HPV 16

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Human vaccine trials using HPV 16 and 18 VLP have shown 100% efficiency conferring type-specific protection against these most prevalent human papillomavirus (HPV) infections and disease. However, protection against 13 high-risk HPV types accounting for additional 30% of cervical cancers remains a challenge. Although peptides of HPV16 L2 minor capsid protein have been identified that induce cross-neutralizing antibodies to HPV6, 11 and 18, L2 is poorly immunogenic in the context of L1/L2 VLP. Thus the aim of this study is to display HPV16L2 epitopes on immunogenic surface loops of L1-VLP, capable of inducing a strong anti-L2 antibody response with cross-neutralizing activity to other mucosal types. Two previously described HPV16L2 epitopes (representing aa 69-81 (A) and 108-120 (B)) were engineered into L1 of BPV1 (between aa residues 133/134). Chimeric proteins were expressed in insect cells, purified on density gradients, and antigenicity was verified by Western blot. By EM chimera B self-assembled efficiently into VLP similar to wt L1, whereas for chimera A mainly pentamers or aggregates were observed. Immunization of NZW rabbits using Freund#s adjuvant induced antisera that recognized GST-HPV16L2 with a titer of 1,000-10,000 by ELISA. Importantly, in a stringent RT-PCR infectivity assay using native HPV 11 virions, antisera induced by chimeric protein A, but not B, neutralized this distantly related type. Immune sera to both chimeric proteins and to peptide B showed significant neutralization of HPV11 virions in a quantitative RT-PCR-assay. In a further infectivity assay using HPV16-pseudovirions, preliminary results show neutralization with anti-peptide A antisera (titer of 200-400) but not anti-peptide B. Neutralization assays using newly developed pseudovirions of high risk HPV18 and 31 are in progress.
Induction of cross-neutralizing antibodies by chimeric L1/L2 VLP may facilitate the generation of broad-spectrum vaccines that protect against a majority of relevant mucosal HPV and associated neoplasia.

P149

TAT-LACK fusion proteins target dendritic cells in vivo and efficiently vaccinate against murine cutaneous leishmaniasis

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In murine cutaneous leishmaniasis healing is associated with IFN-γ production by CD4/Th1- and CD8/Tc1-cells as observed in C57BL/6 mice. Fusion proteins comprised of the protein transduction domain of HIV-1 TAT protein and the Leishmania-antigen LACK translocate proteins directly into the cytosol of DC thus facilitating class I-dependent antigen presentation. TAT-LACK transduced DC efficiently vaccinated against progressive disease in BALB/c and C57BL/6 mice. DC-based vaccinations will not be feasible in Leishmania endemic countries, therefore we tested TAT-LACK in a direct in vivo approach. BALB/c mice were vaccinated i.d. into one ear on d-7 and -6 (10 μg each). In some experiments, 10 μg CpG were injected on d-5 and infections were initiated into the contralateral ear on d0 using physiological low dose inocula with 10E3 L. major. Vaccination with fusion protein was only effective when CpG motifs were co-administered. After vaccination with TAT-LACK, lesions were significantly smaller compared to PBS (50±13 vs. 195±48 mmE3, p≤0.05, wk 10) and fusion protein was superior to injection of LACK alone. Simultaneous injection of CpG together with TAT-LACK on d-7/-6 as well as injection into the unvaccinated ear further decreased lesion volumes (126±15 for original protocol vs. 26±11 for simultaneous injection vs. 40±11mmE3 for unvaccinated ear injection, p≤0.005, wk 9). Infections initiated 2 wks after vaccination compared to 1 wk as described above showed an additional 73% reduction in lesion size (wk 10, p≤0.005), suggesting that longer priming intervals are beneficial for induction of Leishmania-specific T-cells. To investigate whether fusion proteins mediate activation of DC, epidermal sheets were prepared 3 d after vaccination and MHC II+ DC were visualized by immunofluorescence. Both, immunisation with TAT-LACK alone as well as TAT-LACK+CpG resulted in significantly higher activation and migration of DC compared to PBS controls (20.8±2.9 and 24.8±0.3 vs. 2.1±0.3% of total MHC II+ cells, p≤0.05). In summary, our results demonstrate that direct in vivo vaccination using fusion proteins consisting of antigen and TAT requires co-injection of CpG. However, DC activation in vaccinated skin appears to be independent of CpG suggesting that CpG plays a role in later events of the resulting immune response.

P150

Morphotype of Candida albicans has influence over the interaction with dendritic cells

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Although fungal infection caused by rare species of low pathogenic potential becomes more and more important, Candida albicans is a major problem in medical microbiology as a cause of infections in immunocompromised patients. Extensive studies in the mouse model revealed, that Th1 response is protective in candidiasis, whereas a Th2-balanced reaction is not. Dendritic cells have been shown to play a major role in the regulation of a T-cell response towards either Th1 or Th2. It has been shown, that both hyphae and yeast cells of C. albicans are recognized by murine DC. However, whereas yeast induced a protective Th1 response, hyphae inhibited IL-12 secretion and Th1 priming in these cells. In this study, we use pH- and temperature dependent dimorphism for assessing the influence of different morphotypes on the interaction of a virulent C. albicans strain with human DC. Human DCs recognize hyphae and yeast of C. albicans. Yeast are rapidly phagocytosed but start to filament inside the DC, leading to penetration and loss of cellular integrity. Phagocytosis of C. albicans induces large scale reorganistation of the actin cytoskeleton.
The cytokine response induced by Candida is dominated by IL-8 and TNF-a. In contrast to bacteria, only low levels of IL-6 are induced. We could not find a suppressive effect of hyphae on IL-12 secretion. Cytokine inducing factors of C. albicans are cell-bound and not secreted into the medium. Taken together, these data show that morphological plasticity of C. albicans is of impotance in the interaction with human DCs.

P151

L. major-infected B-cell deficient mice show enhanced lesion progression due to impaired CD8 priming and decreased numbers of infected dendritic cells

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Previously, we have shown that dendritic cells (DC) take up L. major parasites in a FcγR-restricted fashion, whereas macrophages phagocytose the parasites via CR3. Phagocytosis in DC leads to activation, antigen presentation in the context of MHC class I/II and IL-12 release. Thus, DC are critical for the induction of Th1/Tc1-dependent protective immunity. We now investigated, if in B-cell-deficient μMT mice altered immune responses against L. major are observed. Wild type C57BL/6 or μMT mice were infected with physiologically relevant low dose inocula of 10E3 L. major. Compared to WT mice, μMT mice showed significantly enhanced lesion progression from week 6 post infection. Within 3 months, no healing was observed. Furthermore, increased lesion sizes in μMT mice correlated with higher parasite burdens: the ears of μMT mice contained greater numbers of parasites reaching a peak load of 7x10E4 parasites/ear at week 6 (wild type mice: 4x10E3, p≤0,05). The IFN-γ/IL-4 ratio of lymph node cells stimulated with antigen was significantly skewed towards Th2 as compared to C67BL/6 mice. No difference was found in the total number of CD11c+ DC that have accumulated in the lesions of μMT mice as compared to C57BL/6 ears. However, lesional ear skin of μMT mice contained significantly fewer L. major-infected DC. Finally, antigen-specific expansion of CD4+ and CD8+ T-cells was detected by CFSE labeling. In μMT mice, decreased numbers of proliferated CD4 cells were observed compared to C57BL/6 cells (2.3±1.2 vs. 12.5±4.7% of total CD4+ T-cells). Interestingly, especially the number of Leishmania-reactive CD8+ T-cells was greatly impaired in the absence of B-cells (2.1±0.7 compared to 19.5±6.6% in WT, n=5, p≤0.05). In summary, enhanced lesion progression in B-cell deficient μMT mice is the result of decreased numbers of infected DC resulting in defects in CD4- and, more importantly, in CD8-priming. IgG-mediated activation of DC might therefore play an important role for T-cell priming in various infectious and tumor diseases.

P152

Identification of RNase 8 as an antimicrobial protein

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Human skin protects itself by the release of antimicrobial proteins. We recently identified the antimicrobial ribonuclease RNase 7 as a major antimicrobial protein of healthy skin. Recently, a novel ribonuclease, termed RNase 8, has been discovered by screening the human genome sequence. Although RNase 7 and RNase 8 share an amino acid sequence similarity of 78 % and a genomic distance of only 15000 bp, it has been demonstrated that RNase 8 in contrast to RNase 7 exhibits no antimicrobial activity against E. coli.
Using real-time PCR we detected gene expression of RNase 8 in primary keratinocytes. Gene expression was moderately induced by Phorbol-12-Myristate-13-Acetate (PMA). To reevaluate a potential antimicrobial function of RNase 8 we established the recombinant production of RNase 8 in E. coli and tested its antimicrobial activity in a microdilution assay system. Consistent with a previous report we detected only low antimicrobial activity against E. coli. However, when testing antimicrobial activity against Staphylococcus aureus, we found potent killing activity in a concentration range below 1 μM. Therefore, RNase 8 seems to be an antimicrobial protein with a preference against gram-positive bacteria like Staphylococcus aureus.
In summary, we identified RNase 8 as a novel antimicrobial protein, which could help to protect human skin and potentially other epithelia against Staphylococcus aureus infection.

P153

Differential roles of IKK1 and IKK2 for C. albicans-induced NF-κB-activation

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Keratinocytes are not only physical barriers between different compartments but are also capable of actively regulating defence mechanisms against microbial pathogens such as Candida. Accordingly, Candida-infected skin revealed a strong up-regulation of the neutrophil-chemoattractant IL-8 as determined by in situ-hybridisation. To further elucidate the molecular mechanisms resulting in skin inflammation we studied the effects of C. albicans (strain SC5314) on different intracellular signalling pathways which mediate the expression of proinflammatory cytokines, chemokines and antimicrobial peptides by keratinocytes. In line with our in situ data, co-culture of C. albicans blastospores with HaCaT keratinocytes at different MOIs resulted in a significant up-regulation of IL-8 expression. Further biochemical analysis revealed that C. albicans induced degradation of IκBα as determined by Western blot analysis. However, activation of the NF-κB signalling module appears to be retarded when compared to TNF-α-induced degradation. To assess the functional relevance of the signalosome we studied keratinocytes which were retrovirally infected to express dominant-negative mutants of IKK1 or IKK2, respectively. Co-culture of cells expressing dominant-negative IKK2 with C. albicans blastospores no longer resulted in up-regulation of IL-8 expression thus confirming the requirement of an IKK2-dependent NF-κB activation pathway. In contrast, expression of dominant-negative IKK1 resulted in partial inhibition of Candida-induced IL-8 expression, while TNF-α-induced IL-8 expression was unaltered in these cells. Another signalling pathway identified to be activated by C. albicans is the p38 mitogen-activated protein (MAP) kinase pathway. We verified its functionality with respect to IL-8 expression by the use of the highly specific inhibitor SB202190. Candida-induced IL-8 expression was dose-dependently blocked. In conclusion, our data demonstrate that C. albicans activates distinct intracellular signalling pathways in human keratinocytes which are necessary for the expression of molecules apparently playing prominent roles in the pathophysiology of Candida infections.

P154

Vitamin D3 inhibits differentiation of monocyte-derived dendritic cells fast and irreversible. A time-kinetic study.

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Dendritic cells seem to be of major importance as regulatory cells driving the psoriatic tissue reaction. The active vitamin D3 metabolite, 1,25(OH)2D3, and its analogues proved to be effective in the treatment of pasoriasis. These compounds have been shown to inhibit the GM-CSF/IL-4-induced differentiation of human monocytes into dendritic cells (MoDC). 1,25(OH)2D3-mediated effects upon DCs are thought to be mediated through the vitamin D receptor (VDR). However, it was recently shown that VDR expression is down-regulated as monocytes differentiate into immature DCs. This suggests that the effect of 1,25(OH)2D3 on MoDC either occurs in the early stage of differentiation or is mediated via a nongenomic pathway. In order to define how 1,25(OH)2D3 regulates DC differentiation, a time-kinetic study was carried out.
MoDC were obtained by incubation of pruified human monocytes with GM-CSF and IL-4 for 5 days. 1,25(OH)2D3 and 24,25(OH)2D3 were dissolved in ethanol. As a marker of differentiation CD1a and CD14 expression was analysed by flow cytometry. Cells were exposed to 1,25(OH)2D3 for various time-points and thereafter removed from the culture medium.
The results showed that a 15 min. exposure to 10-8 mol/L 1,25(OH)2D3 led to significant downregulation of expression of CD1a and upregulation of expression of CD14 as compared to control cells favouring a macrophage-like phenotype. This pattern of modulation was time-dependent and reached a plateau already after 1 hour of exposure. Prolongation of the time of 1,25(OH)2D3 exposure up to 5 days did not result in an increased effect on MoDC CD1a/CD14 expression. The solvent ethanol and 24,25(OH)2D3, a low-affinity VDR vitamin D3 metabolite, were without effect. When MoDC cultured for 3- or 4-days with GM-CSF/IL-4 were exposed to 1,25(OH)2D3 high level expression of CD1a remained unchanged while CD14 was up-regulated.
These findings provide evidence that 1,25(OH)2D3 inhibits MoDC-differentiation only at a very early stage by a VDR-dependent mechanism. This "all-or-nothing" effect was maintained and found irreversible after withdrawal of 1,25(OH)2D3.

P155

Dimethylfumarate inhibits nuclear translocation of NF-kappaB, but not NF-AT in activated human T-cells

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Psoriasis vulgaris is a widespread inflammatory skin disorder in which T-cell mediated immune responses are thought to play a crucial role. Fumaric acid esters proved to be a very efficient systemic treatment for patients with severe psoriasis. Dimethylfumarate (DMF) is a main ingredient of the marketed mixture. It was shown previously that DMF strongly suppresses chemokine production in human keratinocytes and peripheral blood mononuclear cells. Additionally, it was demonstrated that the nuclear translocation of activated NF-kappaB is inhibited in TNF-activated human endothelial cells and fibroblasts. In human T-cells, the influence of fumaric acid esters on the expression of nuclear transcription factors has not been investigated yet.
Therefore, the effects of DMF and its main metabolite methylhydrogenfumarate (MHF) on the nuclear translocation of NF-kappaB and NF-AT in purified human T-cells were assessed. Cells were incubated in medium together with different concentrations of DMF and MHF, ciclosporin or the solvent DMSO alone. An ELISA-based assay quantifying the amount of transcription factors in nuclear extracts was used. Furthermore, electrophoretic mobility shift assays were performed. Our results show that DMF, but not MHF significantly inhibited the translocation of NF-kappaB into the nucleus whereas the translocation of NF-AT was not modulated by DMF or MHF. The data provide evidence that a number of in vitro effects of DMF, namely inhibition of cytokine- and adhesion molecule-expression, can be explained by its inhibitory effects on NF-kappaB. As DMF did not modulate NF-AT and, as previously shown, AP1, it seems that NF-kappaB is a selective target of this compound.

P156

Efomycine M: A "Pan-Selectin" Antagonist?

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The number of skin infiltrating T cells correlates with disease severity in inflammatory skin diseases such as psoriasis or atopic dermatitis. E- and P-selectins are expressed by endothelial cells in inflammatory skin diseases and mediate T cell rolling via their interaction with T cell expressed sialyl LewisX (sLeX) epitopes. Blockade of E- and P-selectin by small molecules is expected to result in a reduction of skin inflammation and, thus, is considered to repesent a promising therapeutic approach. Published data revealed that Efomycine M (Efo-M), produced by Streptomyces sp., significantly exhibits anti-inflammatory activity in two different mouse models of psoriasis by interfering with the binding of E- and P-selectins with their ligands on human T lymphocytes. Here we show that crystallized Efo-M and a liquid state conformation analysis of Efo-M by NMR were in agreement with the known relative configuration of Efo-M. Our data, however, do not provide any evidence that Efo-M and sLeX bind to the same epitope on the selectin surfaces. Moreover, according to Biacore experiments Efo-M binds only weakly to L- and not to E- and P-selectin. In vivo, Efo-M was shown to be effective in vivo in different models of skin inflammation which is in line with recently published data, but effects were only seen if the compound was applied 24 hours before challenge. In contrast, the highest Efo-M concentrations are observed two hours after systemic administration. Our findings argue against a selectin-blocking function of Efo-M and raise questions how Efo-M inhibits inflammatory processes in the skin.

P157

Methylmetacrlyate sections for the analysis of obstructions in long-term subcutaneous suction drainage needles delivering interstitional fluid

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The analysis of interstitional fluids (IFS) continuously collected by suction needles is of interest in pharmacological studies monitoring drug concentrations over several days. Though needles are blocked after some hours, we aimed to learn how to optimize these needles by studying if the blockade is in the needle or in the surrounding tissue and for the material that induces this obstruction.
We constructed a special frame (makrolon, bayer plastics) to stretch pork skin derived from the butcher and explant human skin with subcutaneous tissue derived from spindle edges of routine surgery (informed consent), organ cultured in Dulbecco#s medium (Fa. Biochrom). A low pressure unit delivering 100 mbar (Digital-Baro-Vakuum-Meter) was connected with a prototype blunt steel needle (diameter 0,4mm) with circular holes, fixed in the middle of these tissues (2x2cm) in an angle of 45° for 8 h to measure the IFS flow. Additionally after 8 h the human tissues were studied immunohistochemically by cryostat sections for cytokeratins CK 1,10,16, involucrine (n=2). The tissues with the needle still in it (n=2) were embedded in methylmetacrylate (Technovit 9100 Neu, Kulzer) after several steps during 6 weeks, cut in serial sections perpendicular and parallel to the length axis of the needle (MCP-system, EXACT) and stained with Hematoxylin eosin.
In porc skin (n=6) deliverage of IFS stopped after 2,58 ±0,84 h and in human skin (n=4) after 2,75±1,09 h. Labeling of human cryostat sections showed the epidermal CKs 1,10,16 and involucrine scattered in the deeper dermis near the canal of the needle. Fibrous structures and the tissue fat were found closely adhering to the needle. The cross and longitudinal methylmetacrylate sections showed blood cells in the lumen of the needle.
Porc skin can be used as model to study IFS. The needle transjects keratinocytes in the dermis, but obstruction is induced by blood cells after laceration of small blood vessels and from outside by the tissue attracted by the low pressure. Methylmetarcrylate sections of the needle in tissue allow to study different needle types and the optimal low pressure.

P158

Light Activated Curcumin Induces Apoptosis and Inhibits the EGF-Receptor such as Akt and ERK1/2 in Human Keratinocytes

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Curcuma longa (Zingiberaceae family) is a pharmacologically active plant and spice, widely cultivated in tropical regions of Asia and Central America. After preliminary studies using plant extracts we could show that Curcumin, the yellow pigment of *Curcuma longa*, combined with both, UVA or visible light, induces strong growth inhibition in HaCaT and primary keratinocytes. Investigating the underlying mechanisms it was found that light activated Curcumin induces apoptosis in HaCaT and primary keratinocytes cells. In this context we demonstrated a concentration dependent increase of fragmented cell nuclei, a release of cytochrome c from mitochondria and the activation of caspases 3, 8 and 9 after treatment with Curcumin (0,2-1 μg/ml) plus UVA (1 J/cm²). None of these effects occurred in case of UVA absence. Next, the ability of Curcumin to modulate signalling pathways that support the cell survival was investigated. It was found that the EGF-receptor was inhibited by Curcumin/UVA. Likewise some down stream kinases of the EGF-receptor, as the Akt/PKB kinase and the MAP kinase ERK1/2 were inhibited by Curcumin/UVA in the same manner. In addition, further examinations indicate a concentration dependent inhibition of the NF-κB activity. These results suggest that Curcumin may facilitate apoptosis induction by blocking the EGF receptor and inhibiting the activity of growth-associated kinases. These findings and particularly the observation that Curcumin acts in combination with light (UVA) might contribute to a new photo-therapeutic strategy.

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STAT1 mediated upregulation of transport proteins associated with antigen processing (TAP1 and TAP2) in antigen presenting cells of patients with malignant melanoma receiving INFα treatment

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Although IFNα shows a broad spectrum of immunmodulatory and antiproliferative effects in a variety of malignancies the mechanisms of its antitumor effect and its action in adjuvant melanoma therapy are not completely understood.
In this study we showed that expression of transport proteins associated with antigen processing (TAP1 and TAP2) was significantly upregulated by i.v. intermediate high dose IFNα-treatment in 20 patients with malignant melanoma. This strong stimulatory effect was seen in blood mononuclear cells (PBMCs) both on the RNA level using RT-, Real time- PCR and cDNA microarrays and on the protein level using immunohistochemistry and immunoblotting. Depending on the patient analyzed, 2- to 5-fold upregulation of TAP1 mRNA expression could be detected by Real time-PCR. In vitro studies revealed similar effects or IFNα on monocyte derived dendritic cells, as well as on the THP1 cell line. Pretreatment of THP1 cells with all-trans retinoic acid 16h prior to stimulation with IFNα significantly enhanced the upregulation of TAP1/2. Analysis of signaling molecules in THP1 cells activated by IFNα showed enhanced phosphorylation of STAT1 but not of STAT3, ERK1/2 or p38. Pretreatment of THP1 cells with JAK Inhibitor I 45 min before stimulation with IFNα inhibited upregulation of TAP1/2.
The finding that IFNα stimulates the cytotoxic effector functions in APCs of patients receiving intermediate high dose immunotherapy by enhancing TAP expression and proteasome activity contributes to the understanding of the immunoregulatory role of type 1 interferons and may help to explain the efficacy of IFNα in the treatment of tumors.

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New Phosphodiesterase-4 inhibitors for the treatment of inflammatory skin diseases

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New selective and potent phosphodiesterase-4 (PDE-4) inhibitors with low emetic potential have been examined for their therpaeutic effects in a murine model of allergic skin inflammation induced by the contact allergen, toluene-2,4-diisocyanate (TDI). Female BALB/c mice (20 g) were sensitised to 5% TDI applied topically to stripped abdominal epidermis and were boostered 21 days later with topical administration of 0.5% TDI to both ears. About 7 days later, suitably sensitised animals were distributed to the various treatment groups. Drugs, dissolved in DMSO/acetone (1:1 or 9:1), were administered topically to both sides of the ear (10 μl/surface)either 2 h and 1 h priot to TDI challenge or as a single dosage 2 h before the allergen. Ear thickness was measured just before challenge and 24 h after. All animals were sacrificed immmediately after the final measurement and ear tissue was excised, pulverised under liquid nitrogen, homogenised and processed for IL-4 measurement.
Topically administered cilomilast (1.8 μmol/animal), used as positive control, reduced both ear swelling (92%) and tissue IL-4 synthesis (69%). IBFB 211913 (0.4 μmol/animal) and IBFB 140301 (0.04 μmol/animal)reduced both parameters by approximately 50%. Similar to cilomilast, IBFB 130020 (2.8 μmol/animal) strongly attenuated ear swelling (83%) and IL-4 synthesis (69%)whereas IBFB 150007 reduced both parameters by 76% and 66% respectively albeit used at a lower dosage (1.2 μmol/animal). Histological analysis of skin sections confirmed the positive effects of cilomilast, IBFB 211913, IBFB 130020 and IBFB 150007 on tissue oedema as well as to observe that these PDE-4 inhibitors consistently reduce leukocyte presence in the inflammed skin.
The results show that IBFB#s PDE-4 inhibitors behave similarly to cilomilast in this model of atopic dermatitis. The low emetic potential of the IBFB compounds render them suitable as potential therapeutic agents for the treatment of inflammatory skin disorders.

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Structure - function relation of efomycine family members: Novel insights into the molecular action of small-molecule anti-inflammatory selectin inhibitors

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Recently, we have demonstrated that efomycine M, the lead compound of a novel family of small-molecule inhibitors of E- and P-selectin functions, effectively blocked leukocyte adhesion in vitro, inhibited leukocyte rolling in vivo, and markedly alleviated inflammatory skin disorders in animal models of psoriasis.
In order to assess structural requirements for the inhibition of leukocyte adhesion on the molecular level, we have now generated and characterized several novel members of the efomycine family: The naturally occurring efomycines E and G were purified from Streptomyces BS 1261. Efomycine O was generated by di-O-methylation and efomycine M by defucosylation. Efomycine S was synthesized by macrolide cleavage of efomycine M, and, finally, efomycine T was produced by tetra-O-acetylation of efomycine M.
When leukocyte adhesion to ex vivo vascular endothelium was assessed, it was found that efomycines E, G and M significantly inhibited binding of leukocytes to activated endothelial cells (inhibition by ca. 50% with efomycine E and up to 80% with efomycines G and M), while efomycine O had a somewhat weaker effect that did not reach statistical significance. In contrast, efomycines S and T did not show any inhibitory effects.
Based on molecular modelling and receptor docking studies of efomycines E, M, S and T using state-of-the-art software (4SCAN®, SYBYL) it was shown that the in vivo data regarding leukocyte binding inhibition largely matched the in silico data: The highest docking score (possible receptor-ligand bindings combined with binding energies at specific atomic sites) and the best fitting to the ligand binding domain of E-selectin was demonstrated for efomycine M, while efomycines E and T achieved weaker scores, and efomycine S had the weakest score. Thus, specifically designed molecular modifications of side chains rather than conformational alterations of the pharmacophoric groups shared with the natural selectin ligand, sialylated LewisX, profoundly modulated the biological activities of efomycines.
Our data demonstrate that it is possible, in principle, to generate novel immunomodulatory compounds which differentially affect adhesion of leukocytes, and that their activity characteristics can be simulated in silico.

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A HIGH SPF (25) ONLY DOES NOT PROTECT FROM POLYMORPHIC LIGHT ERUPTION (PLE); ADDITION OF A TOPICAL ANTIOXIDANTAND HIGH UVA PROTECTION SIGNIFICANTLY REDUCES CLINICAL SIGNS OF PLE

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We have recently shown that a new topical antioxidant formulation, containing the nature-derived, modified flavonoid alpha-glucosylrutin (AGR), can reduce UVA-induced oxidative stress in human skin and effectively prevent clinical signs of polymorphic light eruption (PLE) in PLE prone individuals. In our new randomized, double-blind, placebo-controlled clinical trial, we set out to investigate whether the observed beneficial effects could also be observed when tested at a higher SPF level.
Thirty patients with a history of PLE were treated with the following preparations prior to daily photoprovocations: 1. a formulation consisting of 0.25% AGR, 1% tocopherol acetate (vitamine E) and a sunscreen (SPF 25) in a gel formulation (Eucerin®) versus 2. a sunscreen only gel (Ladival®, SPF 25) versus 3. a different sunscreen only gel (ISDIN SA® extreme UVA,) versus 4. placebo (gel base only).
Results after 4 days of daily UVA-irradiations of 60J/cm² to 5x5cm areas to individual predilection sites (upper arms) revealed a statistically highly significant difference ($p<0,001$) between the AGR containing formulation and sunscreen-only containing preparation or placebo, in experimentally eliciting PLE. No patient pre-treated with the antioxidant and sunscreen containing formulation 1 developed clinical signs of PLE while 9 patients (30%) treated with preparation 2 (Ladival®, SPF 25) and 7 patients (23%) treated with (ISDIN SA® extreme UVA,) and 27 patients (90%) treated with placebo, showed typical skin lesions of PLE with concomitant pruritus.
Thus, we would like to suggest that even a sunscreen with a high UVB SPF (25) is not sufficiently effective in abrogating clinical signs of PLE and that combining a potent antioxidant with a highly UVA-protective sunscreen is far more effective in preventing PLE than sunscreen or placebo alone.

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Induction of mitochondrial DNA mutations by treatment of melanocytes with intense pulsed light

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Mutations of mitochondrial (mt) DNA have been shown to play a role in photoaging and carcinogenesis. In addition to this, the effective removal of pigmented skin lesions by lasers or intense pulsed light (IPL) technology is increasingly applied in clinical practice. Lasers and IPL employed for the removal of pigmented skin lesions emit radiation within the interaction spectrum of melanin, thus inducing selective photothermolysis. While progress has been made in the identification of the mechanism of action by which melanin is removed by lasers or IPL, little is known about long term effects of IPL interaction with melanocytes. Since melanin and DNA absorb the same wavelengths, we investigated whether treatment of melanocytes by IPL technology leads to the induction of mtDNA mutations. Dose response experiments did not reveal decreased viability or induction of apoptosis in melanocytes following exposure to IPL at doses regularly applied for treatment of pigmented skin lesions (0-14 J/cm²). In addition to this, viability of melanocytes was normal for up to 7 days. Real time PCR did not reveal mutations of mtDNA after 24 h. However, timepoints later than 24 h did show increased levels of mtDNA deletions reaching a maximum at four days. Follow-up of mtDNA deletions revealed a subsequent decrease of induced mutations leading to levels undetectable by real time PCR seven days after IPL exposure. These data indicate that exposure of melanocytes to IPL at doses regularly applied for removal of pigmented skin lesions do indeed induce photoaging- and carcinogenesis-associated mutations of mtDNA while viability of cells is unaffected.

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Sun protection and vitamin-D deficiency in renal transplant recipients.

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Immunosuppressive therapy markedly increases the risk to develop UV-induced skin cancer. Therefore, renal transplant recipients need to protect themselves against UV-light. However, it has been suggested that lack of UV-exposition may induce vitamin D deficiency. This represents a serious dilemma, for a connection between vitamin D deficiency and other severe health problems including various types of cancer has now been demonstrated. We aimed to test the hypothesis whether renal transplant recipients are at risk to develop vitamin D deficiency. Basal 25(OH)D serum levels were analyzed in renal transplant patients (n=31) and in an age- and gender-matched control group at the end of wintertime (February/March). All renal transplant patients had been advised to avoid UV-exposition by mechanical protection and to use sunscreens after transplantation. Basal 25(OH)D levels were compared using a non parametrical test (Wilcoxon rank sum test). $P < 0,05$ was considered significant. Basal 25-hydroxyvitamin D serum levels were significantly lower in renal transplant recipients as compared to controls (p: 0,007). Geometric mean (+ standard error of the mean) in renal transplant patients was 10,85 ng/ml compared to 25,05 ng/ml in the control group. Our findings demonstrate that renal transplant recipients are at high risk to develop vitamin D deficiency, that has to be substituted (e.g. via oral treatment) to protect sufficiently against serious vitamin D deficiency-related health problems without increasing the risk to develop UV-induced skin cancer. These connections should as well be considered in other groups at high risk to develop vitamin D deficiency due to lack of sun exposure, e.g. in patients with xeroderma pigmentosum, and when developing recommendations for sun exposure in skin cancer prevention programs.

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Interleukin-12 prevents UV-induced immunosuppression via induction of DNA repair

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Interleukin (IL)-12 prevents the suppression of contact hypersensitivity (CHS) by ultraviolet radiation (UV). In addition, IL-12 is able to break UV-induced tolerance which is mediated via regulatory T cells (Tr). Recently, IL-12 was found to reduce UV-induced DNA damage most likely via the induction of nucleotide excision repair (NER). UV-induced DNA damage, in particular cyclobutane pyrimidine dimers (CPD), is the crucial molecular trigger of UV-mediated immunosuppression, Thus, we initiated studies into immune restoration by IL-12 to discern whether its effects are linked to DNA repair. To address this issue, we utilized Xpa knock out mice (Xpa-/-) which are completely deficient in NER. IL-12 prevented both UV-induced suppression of the induction of CHS and depletion of Langerhans cells (LC) in wild type (WT), but not in Xpa-/- mice. In addition, IL-12 did not prevent the development of UV-induced Tr in Xpa-/- mice. In contrast, IL-12 was able to break established UV-induced tolerance and inhibited the activity of Tr in both strains, indicating these effects to be independent of NER. There is evidence that UV-induced DNA damage is the major molecular trigger for the emigration of LC to the draining lymph nodes. It also impairs their capacity to present antigen, which in turn results in the lack of sensitization and the induction of tolerance. Thus, we determined whether IL-12 reduces the number of CPD-positive LC in the draining lymph nodes. FACS analysis revealed an increase of cells staining for the LC- specific marker Langerin and for CPD in the lymph nodes of UV-exposed mice both in WT and Xpa-/- mice. Injection of IL-12 drastically reduced the number of LC staining for CPD. In contrast, IL-12 did not have an effect on the frequency of CPD-positive LC. This indicates that the reduction of CPD in LC by IL-12 is critically dependent on functional NER. Together, the data suggest that the ability of IL-12 to prevent UV-induced immunosuppression may be due to its capacity to remove UV-induced DNA damage via induction of NER.

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Real time in vivo cytokine profile in human dermis in UVB induced inflammation by microdialysis technique reveals rapid cytokine kinetics

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Ultraviolet B (UVB) irradiation dose-dependently affects the immunity of the skin. Whereas lower doses initiate immunosupression, the application of higher doses induce sunburn and are proposed to be involved in processes leading to skin aging as well as photocarcinogenesis. The immune response developing after high-dose UVB-irradiation is triggered by cytokine release into the extracellular space. It has been a matter of debate which cytokines might be the major effectors for the acute and long-term effects of UV-irradiation. In this regard the so far lacking possibility to measure multiple cytokines in an in vivo setting was a crucial limitation. Further, it becomes more and more obvious, that the simplification of the TH-1/TH-2 model can not cope the in vivo situation. Therefore, we aimed to establish a method, that allows us to determine the in vivo cytokine profile during the initiation of a mild, UVB-induced sunburn. The applied methods combine the advantages of low skin-irritation and the possibility of long-term measurements directly on the UV-application site. Further, they allow profiling of multiple cytokines on a protein level, which is superior to RNA-based methods, since cytokines are often released from preformed intracellular stores after stimulation. Intradermal microdialysis was applied over a time period of 48h to collect all secreted proteins up to a size of 100kDa. Analysis of the microdialysis fluid on microarray protein chips indeed revealed that UVB-irradiation induces a strong and rapid inflammatory response, mainly depending on the upregulation of TNF-a, IL-6 and IL-8, but also showing characteristics of both TH1 and TH2 immune-responses by release of IL-2, IL-12, IL-4, IL-10, TGF-b1. After 24h the cytokine profile changed towards a TH2 response (IL-4, IL10). Interestingly, also TH1 associated angiostatic chemokine CXCL10 (IP-10) is stronly elevated. Our results show, that the combination of the two novel methods of intradermal microdialysis and protein microarray analysis enable us to get a more realistic picture of the complex and rapidly changing interstitial cytokine milieu in UVB-irradiated human skin.

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Overexpression of Phospholipid Hydroperoxide Glutathione Peroxidase in Human Dermal Fibroblasts Abrogates UVA-induced Expression of Interstitial Collagenase/Matrix-Metalloproteinase-1 by Suppression of Phosphatidylcholine Hydroperoxide-mediated NFkappa B Activation and Interleukin-6 Release

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Phospholipid hydroperoxide glutathione peroxidase (PHGPx) reveals high specific activity in reducing phosphatidylcholine hydroperoxides (PCOOH) and, thus, may play a central role in protecting the skin against UV-triggered long-term effects like cancer formation and premature skin aging. We addressed the role of PHGPx in the protection against UV-induced expression of matrix-metalloproteinase-1 (MMP-1). Therefore, we have created human dermal fibroblast cell lines overexpressing human PHGPx that showed a significant increase in PHGPx activity. In contrast to a maximal 4.5-fold induction of MMP-1 mRNA levels in vector-transfected cells at 24 h after UVA-irradiation, no MMP-1 induction occurred at any studied time point after UVA treatment of PHGPx overexpressing fibroblasts. As interleukin-6 (IL-6) was shown to mediate the UVA-induction of MMP-1, we studied whether PHGPx overexpression might interfere with the NFkB-mediated IL-6 induction and downstream signaling. Using transient transfections of IL-6 promoter constructs containing NFkB binding sites, we observed a high induction of the reporter gene luciferase in vector-transfected control cells and a significantly lower induction in PHGPx-overexpressing fibroblasts following UVA irradiation. Both UVA irradiation and treatment of fibroblasts with PCOOH led to phosphorylation and nuclear translocation of the p65 subunit, whereas cells overexpressing PHGPx exhibited impaired NFkB activation, p65 phosphorylation and nuclear translocation. The PHGPx-overexpressing fibroblasts showed a reduced constitutive, UVA, and PCOOH induced IL-6 release. This, together with the suppression of UVA-induced IL-6 release in the presence of Trolox, a chain breaker of PCOOH-initiated lipid peroxidation, indicates that UVA-induced PCOOH and subsequent lipid peroxides initiate the NFkB-mediated induction of IL-6, which mediates the induction of MMP-1. Our finding is particularly relevant in the light of already available mimetics of PHGPx.

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In vivo relevance of infrared A radiation induced Matrixmetalloproteinase-1 expression: differential effects on epidermis and dermis

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Infrared-A(IR-A) radiation is the major part of natural sunlight and also emitted by artificial irradiation devices which are increasingly used for reasons of lifestyle or therapeutic purpose, despite the fact that there is evidence that IR-A causes premature skin ageing. As we have shown previously IR-A has a significant impact on living cells in vitro: increased generation of reactive oxygen species, activation of MAP-Kinases and subsequent upregulation of Matrixmetalloproteinase-1 (MMP-1) are consequences of low, physiological doses of IR-A irradiation. Here we provide first evidence from in vivo studies, that IR-A irradiation leads to a MMP-1 upregulation in human skin as well. Buttock skin of healthy volunteers was irradiated with IR-A at doses corresponding to 3-6 hours of summer sunlight exposition (360 - 720 J/cm²). After 24 hours 4mm punch biopsies were taken from the irradiated area and a sham irradiated control area and analyzed. Assessment of the MMP-1 mRNA level in the skin samples revealed a strong upregulation (up to 15-fold) in all cases, displaying interindividual differences. Separation of epidermis and dermis utilizing the salt split technique disclosed that the upregulation of MMP-1 mRNA occurs exclusively in the dermis, while the epidermal level stays unaltered. The corresponding MMP-1 inhibitor TIMP-1 was only affected slightly in both tissues. Extending our analysis to immunohistochemical examinations confirmed our observation: a strong upregulation of MMP-1 protein with an unaltered level of TIMP-1 protein level in the dermis occurred after IR-A irradiation while the epidermal levels of MMP-1 and TIMP-1 both were comparable to the sham irradiated skin. In conclusion, we here show for the first time, that IR-A radiation in vivo leads to a shift of the MMP-1/TIMP-1 balance in the skin, manifesting exclusively in the dermis. These findings underline the suggested role of IR-A in premature skin ageing.

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Functional analysis of the role of the raft-associated protein Caveolin-1 (Cav-1) in Ultraviolet A radiation (UVAR)-induced signaling in human keratinocytes (KC).

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Rafts are cell membrane microdomains which serve as platforms to transfer extracellular signals into a cell. Raft signaling critically depends (i) on the lipid composition of these microdomains which are rich in sphingomyelin (SM) and cholesterol and (ii) on the function of raft-associated proteins such as Cav-1. We have previously provided evidence that raft signaling is important for UVAR-induced signaling in KC, because modulation of membrane cholesterol content affected their capacity to mount a UVA response. In addition, raft-associated proteins may be involved, because UVAR decreased the Cav-1 content in the raft, but not in the non-raft fraction of KC cytoplasmic membranes. We now report that upon UVA irradiation, Cav-1 is transiently phosphorylated at Y14 within the cytoplasmic membrane of primary human KC. This phosphorylation (P) of Cav-1 is caused by the non-receptor protein tyrosine kinase src as was shown by western blotting using the src inhibitor Su6656. UVA-induced translocation of Cav-1 from the raft fraction into the non-raft fraction of the cytoplasmic membrane required Cav-1 P, because it could be inhibited upon treatment of KC with Su6656. We have previously shown that UVA-induced signaling in KC is initiated by the formation of second messenger ceramide (Cer) from cell membrane SM (EMBO J 19:5793, 2000). Interestingly, preincubation of KC with Su6656 did not prevent UVA-induced Cer formation, but completely blocked P of ERK1/2 and p38 MAPkinases in irradiated KCs, indicating that Cer formation occurs upstream and MAPKinase activation downsteam of src kinase P in irradiated KC. Most importantly, Cav-1 P, but not MAPkinase activation were of functional relevance for UVAR-induced gene expression, because Su6656 treatment resulted in complete inhibition of UVAR-induced ICAM-1 expression, whereas preincubation with the cell permeable inhibitor of p38 MAPkinase e.g. SB203580 and of ERK1/2 e.g. PD98059 had no effect on UVA-induced gene expression. Our studies demonstrate for the first time a functional role for Cav-1 P in UVAR-induced signaling and indicate that UVA-induced gene expression of human KC critically depends on activation of the raft associated src kinase.

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The efficacy of excimer laser (308nm) for vitiligo at different body sites

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Many clinical trials have been performed on different treatment modalities for patients with vitiligo with more or less success. Recently, the treatment with XeCl-excimer laser generated 308-nm UVB radiation has shown promising results in the treatment of patients with vitiligo.
In this controlled, prospective trial we studied the efficacy and patients satisfaction of XeCl-excimer laser for treatment of vitiligo patches at different body-sites and evaluated the achieved repigmentation 12 months after the end of therapy.
Twenty-five patients with generalized or localized vitiligo with a total of 85 lesions on different body sites were enrolled in this study. One vitiligo lesion was randomly selected for treatment per diseased body-site (face, trunk, arm, elbow, wrist, dorsum of the hand, finger, leg, knee, dorsum of the foot) per patient. Vitiligo patches were treated with 308-nm XeCl-excimer laser 3 times a week for 6 to 10 weeks. The overall repigmentation grade of each treated lesion was evaluated once a week on a 5 point scale rating from 0 (no repigmentation), 1 (1 - 5%), 2 (6 - 25%), 3 (26 - 50%), 4 (51-75%), to 5 (76 - 100%), respectively.
Twenty-four patients completed the study. Within 6 to 10 weeks of treatment 67% of the patients (16/24) and 60% (48/80) of the treated vitiligo lesions developed follicular repigmentation of at least one of their vitiligo lesions whereas 8 patients did not show repigmentation in any of their lesions. Lesion repigmentation started after a mean of 13 treatments in lesions located on the face, trunk, arm, and leg (high-responder location), and after a mean of 22 treatments in lesions located on the elbow, wrist, dorsum of the hand, knee, and dorsum of the foot (low-responder location). Untreated control lesions and lesions located on the fingers did not achieve any repigmentation. After 10 weeks of treatment repigmentation of more than 75% was found in 25% (7/28) of lesions of the high-responder location group versus 2% (1/43) of lesions of the low-responder location group. In most cases, laser repigmentation was resistant as determined 12 months after the end of therapy.
308-nm excimer laser is an effective modality for the treatment of vitiligo. However, similar to other non-surgical treatment modalities, the therapeutic effect is mainly dependent on the location of vitiligo lesions.

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Keratinocytes-derived Vascular Endothelial Growth Factor protects skin from UVB-induced photo-damage by promoting blood vessel survival

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Exposure of skin to ultra-violet light can induce erythema, and in extreme cases, edema, suggesting that these reactions are part of a protective response to the damaging effects of the irradiation. Vascular Endothelial Growth Factor (VEGF), a potent endothelial cell mitogen and blood vessel permeability factor, is upregulated in epidermis in vivo, and in primary keratinocytes (KC) in vitro after UVB exposure. We have previously shown that mice in which VEGF has been inactivated in epidermal KC (VEGF-AΔK5-cre/ΔK5-cre), but not control mice, develop superficial wounds after a high dose of UVB irradiation. Such mutant mice produce only low amounts of VEGF in the epidermis after UVB irradiation, compared to the significant upregulation of this factor in control epidermis. Histologic examination of the irradiated skin revealed an increase in the number of Tunel positive KC in mutant mice compared to controls 24 hours after UVB exposure. However, UVB irradiation did not induce more apoptosis in cultured KC from mutant mice compared to controls, suggesting that they are not per se more sensitive to UVB-induced cell death. Our previous work had shown that blood vessel response was impaired in the mutant mice both after a single high dose and chronic low dose UVB irradiation. Since VEGF has been implicated as a survival factor for endothelial cells exposed to stress stimuli such as TNF-a, H202 and serum starvation, we directly investigated the effect of VEGF on UVB induced cell death in endothelial cells in vitro. We found that VEGF dose- and time-dependently protected human dermal microvascular endothelial cells (HDMVEC), but not cultured primary murine KC to UVB induced apoptosis. Taken together our results suggest that VEGF, produced by epidermal KC, maintains the integrity of UVB-irradiated skin by not only promoting expansion of the dermal vascular plexus, but also by directly acting as a blood vessel endothelial cell survival factor.

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Phospholipid oxidation products induce expression of the anti inflammatory protein heme oxygenase 1 in dermal fibroblasts and keratinocytes.

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A specific class of phospholipid oxidation products i.e. oxidized L-alpha-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholin (OxPAPC) that have been found in cell membranes of apoptotic cells, oxidized low-density lipoproteins, and at sites of inflammation, have recently been shown to directly act as potent inhibitors of acute, LPS-dependent inflammation. In addition, it was demonstrated that OxPAPC induces expression of Heme Oxygenase-1 (HO-1), an enzyme with potent anti-inflammatory properties, adding an unrelated mechanism by which oxidized phospholipids may influence inflammatory reactions.
When we studied the effects of lipid oxidation products on primary keratinocytes, dermal fibroblasts, HACAT and in an epidermal equivalent model, we found that OxPAPC induced expression of both HO-1 mRNA and protein. Addition of the radical scavanger bHT (butylated hydroxytoluene) did not inhibit the induction of HO-1 expression, indicating this process was not dependent on putative oxidative properties of OxPAPC. It has been shown that also UVA irradiation is a potent inducer of HO-1 expression, but also of the proinflammatory mediator cyclooxygenase-2. We show here that induction of HO-1 expression by OxPAPC does not lead to concomitant expression of this proinflammatory mediator. We also could show that irradiation of PAPC with UVA (340-390nm) leads to formation of oxygenated and oxidized forms of PAPC (as identified by mass spectrometry), including compounds that are known to induce HO-1 expression. While it was assumed that UVA- oxidation of membrane phospholipids can lead to HO-1 induction, we here show for the first time that UVA oxidation of a specific phospholipid leads to the formation of one or several compound with this biological activity.
Since HO-1 has been implicated to play a role in wound healing by degrading free heme and thereby preventing free radical formation and lipid peroxidation, our data identify one possible mechanism for UVA induced HO-1 expression and suggest that HO-1 induction by oxidized phospholipids represents a potential new therapeutic approach in the treatment of skin diseases associated with tissue damage mediated by oxidative stress.

P173

Ultraviolet-B induced alterations in nuclear hormone receptor expression in cultured human keratinocytes

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In this study we determined if UV-B irradiation alters the expression of nuclear hormone receptors and their ability to transactivate response elements in their target genes. mRNA levels of PPAR-alpha and LXR-alpha were decreased by 50% 24 hours after UVB treatment of primary cultured keratinocytes. As previously reported retinoid X receptor (RXR)-alpha and vitamin D receptor (VDR) were also decreased 24 hours after UVB exposure. In contrast PPAR-delta was increased, and LXR-beta, CAR, and TRs were unchanged. In this experiment PPAR-gamma was not detectable on Northern blots in cultured keratinocytes. Time course studies revealed that these decreases occurred as early as 6 hours after UVB exposure. In addition, mRNA levels of acetyl-CoA-oxidase, a target gene for PPAR-alpha, and ATP-binding cassette transporter (ABC)-1, a target gene for LXR-alpha, were similarly decreased after UVB exposure. Furthermore, cultured keratinocytes transfected with a PPAR or LXR response element showed a marked decrease in promoter transactivation following UV-B irradiation. This decrease was not restored by either ligand treatment, over-expression of the receptor, or the combination of the two. Finally, we also observed down regulation of DRIP, suggesting that the inhibition of gene expression by UV light is secondary to not only decreases in PPAR and LXR alpha but also to post receptor mechanisms that link the binding of transcription factors to DNA with increased transcription. The UV-induced down regulation of DRIP, PPAR-alpha, LXR-alpha results in the decreased expression of downstream target genes that could reduce the movement of cholesterol out of cells and the oxidation of fatty acids.

P174

Identification of new components of the cJun/HIF transcription complex in melanoma cells

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Hypoxia is a critical factor for tumor progression and metastasis. The molecular mechanisms underlying this process are, however, poorly understood. Hypoxia induces the expression of a variety of genes, such as angiogenesis factors and enzymes involved in glucose metabolism. Moreover, in recent years hypoxia-inducible transcription factors have been identified. Among these, HIF-1á/ARNT and cJun play an outstanding role. In the present report, we were able to demonstrate by use of electrophoretic mobility shift assays (EMSAs) that cJun and HIF-1á DNA binding are induced in melanoma cells under hypoxia. Moreover, it could be shown that cJun and HIF-1á directly interact. To identify further molecules that may contribute to this central complex of gene transcription, immunoprecipitation experiments were performed in malignant melanoma cells using an antibody against cJun. Co-immunoprecipitating proteins were identified by a combination of polyacrylamide gel electrophoresis (SDS-PAGE) and Maldi-TOF and Q-TOF mass spectrometry. These results were confirmed by Western Blotting. By this means, a series of interesting new components of the cJun/HIF transcription complex could be identified. Among these were heat shock protein (hsp) 90, hsp 70, nucleophosmin and GRP78. Taken together, our analyses identified new members of the cJun/HIF transcription complex in malignant melanoma. These molecules might serve as targets for a direct interaction with hypoxia-induced gene expression in this tumor.

P175

Chemically distinct anticoagulants exert differential effects on experimental melanoma metastasis

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Metastasis remains the major cause of mortality in cancer patients, constituting a key therapeutic challenge. Accumulating evidence indicates that metastasis can be effectively inhibited by the widely used anticoagulant heparin in different tumor models. We have previously shown that the anti-metastatic effects by heparin are due to its action on both endothelial and platelet P-selectin-mediated interactions. We hypothesised that the ability of anticoagulants to exert anti-metastatic effects in vivo is dependent on their ability to inhibit P-selectin-dependent binding. We therefore examined two different fractionated low molecular weight heparins (enoxaprin, nadroparin) and one pentasaccharid (fondaparinux) in relation to unfractionated heparin (UFH) as to their effects on P-selectin-dependent binding in vitro and as to their antimetastatic effects on B16.F10 melanoma lung metastasis model in vivo. Binding of a P-selectin fusion protein to leukocytes, which had been stimulated to express ligands for selectins, was analysed by flow cytometry. Only UFH and nadroparin had an impact on P-selectin binding; while enoxaprin and fondaparinux had no significant effect (P-selectin binding (%): unstimulated leukocytes 33±15; stimulated 50±13; UFH 28±12; nadroparin 24±11; enoxaprin 36±13; fondaparinux 41±16). We next evaluated the impact of these different anticoagulants on melanoma metastasis using the B16.F10 model. In concordance with our in-vitro results, only UFH and nadroparin reduced melanoma metastasis significantly even at low doses (20IU/mouse; number of lung colonies: NaCl 361±55; UFH 116±62; nadroparin 129±70; enoxaprin 315±82; 332±115). While at higher doses (60IU/mouse) enoxaprin was also effective. Our data indicate that P-selectin inhibition is relevant for preventing melanoma metastases. Clinical trials using anticoagulants in cancer patients should primarily focus on those with a P-selectin inhibitory effect.

P176

Lowering of Tumour Interstitial Fluid Pressure in Squamous Carcinomas and Melanomas Enhances the Uptake of Therapeutics in a Xenograft Tumour Model

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Tumour interstitial fluid pressure (TIFP) is a biological factor that prevents the adequate uptake of therapeutics of macromolecular range in tumour tissue. In normal tissues the pressure is around 0 mbar but in solid tumours it is much higher. This hypertension leads to a pressure gradient between tumour tissue and host tissue resulting in the "squeeze-out" of interstitial fluid into the surrounding tissue, thus flushing drugs out from the tumour. In the present study the impact of TIFP on the delivery of potential therapeutics was investigated by intravenously injected fluorescent-coupled dextrans as marker molecules for potential cytostatics in a xenograft tumour model.
Small pieces of A431 squamous epidermoid vulva tumours were subcutaneously transplanted or G361 melanoma cells were injected into the left and right back of anaesthetised NMRI nude mice. After 4-5 weeks (A431) / 5-6 weeks (G361) the tumours were implanted with a medical mini-redon drain system to lower TIFP.
In our experimental set-up we could demonstrate that the reduction of TIFP to 0 mbar for a timespan up to 36 h by drainage enhanced the delivery of fluorescent-coupled dextrans. Pictures of cryosectioned tumour tissue, done by fluorescent microscopy and confocal laser scanning microscopy, showed an enrichment of dextrans in tumours with reduced TIFP in contrast to untreated control tumours. Furthermore, Western blot analysis of tumor tissue showed a reduction of p44/42 MAPK phosphorylation in drained tumors. These data demonstrate that lowering of TIFP can augment the delivery of dextrans to tumours. Therefore, the reduction of TIFP could enhance the efficacy of anti-tumour therapies using chemotherapeutics.

P177

Early TCR-β and TCR-γ PCR detection of T-cell clonality indicates minimal tumor disease in lymph nodes of cutaneous T-cell lymphoma: diagnostic and prognostic implications

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The lymph nodes in cutaneous T-cell lymphoma (CTCL) are generally the most early extracutaneous manifestation and is relevant for prognosis, however, their involvement is difficult to assess. Aim of our study was to define the diagnostic and prognostic value of T-cell clonality analysis for more precise assessment of lymph node involvement in CTCL. After identifiying the malignant T-cell clone in skin we investigated the presence of T-cell clonality in palpable lymph nodes of 36 patients with CTCL, as related to the histological findings and the clinical outcome. T-cell clonality was determined by two independent methods, using a recently developed TCR-β PCR technique and also TCR-γ PCR. In addition, the PCR products were analyzed by Genescan-analysis and DNA-sequencing. T-cell clonality was found in 22/22 lymph nodes with CTCL-involvement and in 7/14 non-involved dermatopathic lymph nodes. The clonal T-cell populations found were in all cases identical to those detected in the corresponding skin lesions, identifying them as the tumor cell population. In none of 12 dermatopathic lymph nodes of 12 patients with inflammatory skin diseases T-cell clonality was found.
The detection of a clonal T-cell populations in 7/14 lymph nodes designed as dermatopathic was associated with a limited duration of patient#s survival (74 months, CI: 66-82 months), similar to patients with histologically confirmed lymph node involvement (41 months, CI: 35-47 months), in contrast to patients without T-cell clonality (all 7 patients were alive at the time of the last follow-up). Thus, T-cell clonality analysis is an important adjunct in differentiating benign dermatopathic lymphadenitis from early CTCL involvement, and should be taken into consideration in the process of staging of patients with CTCL.

P178

Changes in oncogene expression and Rb phosphorylation during the progression of Mycosis fungoides (MF) and Sézary syndrome (SS)

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Mycosis fungoides (MF) and its leukemic variant the Sézary syndrome (SS) are the most frequent types of cutaneous T cell lymphomas (CTCL) and their aetiology is quite unknown. They progress from a patch/plaque stage to a tumor stage that often kills the patients. Such stage transitions are accompanied with changes in oncogene expression and modifications of tumor suppressor genes. We studied the retinoblastoma (Rb) tumor suppressor gene that regulates the cell cycle and is inactivated by phosphorylation and the ras and bmi-1 oncogenes. The ras oncogenes are important for the maintenance of the transformed phenotype and Bmi-1 is an inhibitor of the expression of p16 another cell cycle regulating gene and its expression could explain the absence of p16 in MF and SS cells.
To determine the expression and phosphorylation of the Rb gene we tested the MF cell line MyLa and the SS cell lines Hut78, SeAx and HH ba Western blotting and phosphorylation-specific Rb antibodies. Biopsies from MF and SS skin lesions were tested with the same antibodies by immune histochemistry. Ras and Bmi-1 proteins in the MF and SS cell lines were also detected by Western blotting and by immune histochemistry in MF and SS skin lesions.
In all 4 cell lines we found that Rb was either phosphorylated or not expressed, indicating that Rb is either inactive or absent in these cell lines. In the skin lesions we found that phosphorylated Rb was much more prominent in the late (tumor) stage than in the early (patch/plaque stage). Ki-ras and bmi-1 were expressed in the four cell lines and their expression was also much more prominent in the tumor stage. Silencing of the cell cycle regulator p16 during CTCL progression has been ascribed to methylation in 30-60% of the tested cases. Our data indicate that bmi-1 activation may be an additional way of p16 inactivation in CTCL.
Taken together our data imply that oncogenes are activated and tumor repressor genes are inactivated during the progression of MF and SS.

P179

Downregulation of RPE65 - a possible receptor for plasma retinol binding protein - during squamous skin carcinogenesis

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Background: In a recent report, we described RPE65, a protein originally characterized in retinal pigment epithelium, to be expressed in normal human epidermis. RPE65 is suspected to be involved in cellular uptake of retinol which is transported in the bloodstream complexed with plasma retinol-binding-protein (RBP).
Objective: The aim of the present study was to evaluate protein and mRNA expression of RPE65 in actinic keratosis (AK), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) compared to normal patient#s skin.
Methods: RPE65 mRNA expression in skin tumours relative to normal skin of the respective donor was studied by real time PCR in AK (n=15), invasive SCC (n=30) and in BCC (n=18). For immunohistochemical staining of formalin-fixed and paraffin-embedded tissue sections a peptide specific anti-RPE65 antibody was used to study protein expression of RPE65.
Results: RPE65 mRNA expression was progressively reduced from premalignant AK to invasive SCC. Immunohistochemistry revealed a continous staining of basal and suprabasal keratinocytes in normal human epidermis. Protein expression of RPE65 in AK shown by immunohistochemical staining was reduced and irregular whereas invasive SCC revealed no staining of tumour cells with the anti-RPE65 antibody. RPE65 mRNA values were elevated in BCC whereas immunohistochemical staining for RPE65 protein of various BCC showed positive as well as negative tumour cells.
Conclusions: We find progressive downregulation of the RPE65 receptor molecule during squamous skin carcinogenesis.

P180

Analysis of mechanisms that modulate the growth response of melanoma cell lines to vitamin D analogs in vitro

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Vitamin D analogs inhibit proliferation and induce differentiation in various cell types, including human melanocytes. For unknown reasons however, some tumor cell lines fail to respond to antiprolifferative effects of these compounds. We studied effects of 25(OH)D3, 1,25(OH)2D3, and vitamin D analog seocalcitol (EB 1089) on the growth of seven melanoma cell lines (IGR, MelJuso, MeWo, SK-Mel-5, SK-Mel-25, SK-Mel-28, SM). As measured by a WST-1 based colorimetric assay, three melanoma cell lines (MeWo, SK-Mel-28, SM) responded to antiproliferative effects of active vitamin D analogs, while the others were resistant. A strong induction (up to 7000-fold) of 1,25-dihydroxyvitamin D-24-hydroxylase (24OHase) mRNA was detected in responsive cell lines along with 1,25(OH)2D3-treatment, indicating functional integrity of vitamin D receptor (VDR)-mediated transcription. In contrast, induction of 24OHase was much lower in resistant melanoma cells (up to 70-fold). VDR mRNA was induced up to 3-fold both in responsive and resistant cell lines along with 1,25(OH)2D3-treatment. Antiproliferative effects of calpain inhibitors were comparable in responsive and resistant melanoma cells. Expression of mRNA for vitamin D-activating enzymes vitamin D-25-hydroxylase (25OHase) and 25-hydroxyvitamin D-1α-hydroxylase (1αOHase) was detected in all melanoma cell lines analyzed. Expression of these enzymes was only marginally modulated along with vitamin D analog treatment. Proliferation of melanoma cells was not modulated by treatment with 25(OH)D3, indicating no significant increase in the endogeneous production of antiproliferative acting 1,25(OH)2D3. Interestingly, we identified different alternate splice variants of the 1αOHase gene, their expression pattern was not modulated by treatment with 25(OH)D3 or 1,25(OH)2D3. In conclusion, our results indicate that at least a proportion of cases with metastasizing melanoma represents a promising target for palliative treatment with new vitamin D analogs that exert little calcemic side effects and for pharmacological modulation of 1,25(OH)2D3-synthesis/metabolism.

P181

The vitamin D system represents a key regulator of the growth of cutaneous squamous cell carcinomas (SCC).

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Increasing evidence points at an important function of vitamin D metabolites for growth regulation in various tissues, and new vitamin D analogs are interesting candidates for the treatment of malignancies, including SCC. We have analyzed expression of vitamin D receptor (VDR), vitamin D-25-hydroxylase (25-OHase), 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase), and 1,25-dihydroxyvitamin D-24-hydroxylase (24-OHase) in cutaneous SCC and SCC cell lines. Intensity of VDR-immunoreactivity was increased in SCCs as compared to normal human skin (HS). VDR-staining did not correlate with histological type or grading, nor with markers for proliferation, differentiation or apoptotic cells. SCC cell lines (SCL-1, SCL-2) revealed VDR-immunoreactivity in vitro and incubation of these cells with calcitriol resulted in a dose-dependent suppression of cell proliferation (up to appr. 30%), as measured by a WST-1 based colorimetric assay. RNA levels for VDR, 25-OHase, 1α-OHase, and 24-OHase were significantly elevated in SCCs as compared to HS, as measured by real-time PCR. In conclusion, our findings demonstrate that alterations in VDR expression as well as in local synthesis or metabolism of vitamin D metabolites may be of importance for growth regulation of SCCs. Additionally, SCCs represent potential targets for therapy with new vitamin D analogs that exert little calcemic side effects or for pharmacological modulation of calcitriol synthesis/metabolism in these tumors.

P182

Implications for an important function of clusterin for pathogenesis and progression of malignant melanoma.

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Clusterin is a glycoprotein that is implicated in various cell functions including cell growth, cell adhesion and apoptosis. There are two known clusterin isoforms that are obtained by alternate splicing, the nuclear (nCLU) and the secretory (sCLU). The pro-apoptotic nCLU has been shown to be involved in the regulation of cell-cycle progression and apoptosis. We have analyzed immunohistochemically paraffin sections of primary cutaneous malignant melanomas (n=25), metastases of malignant melanomas (n=18) and acquired melanocytic nevi (n=30) using clusterin specific antibodies that detect proapoptotic nCLU and secretory sCLU and a streptavidin-peroxidase technique. Both the proapoptotic nCLU and the antiapoptotic sCLU were detected in a proportion of malignant melanomas and metastases of malignant melanoma, but not in acquired melanocytic nevi. Additionally, we have analysed expression of clusterin in various melanoma cell lines (MeWo, SkMel28). All melanoma cell lines analysed revealed strong expression of clusterin mRNA and protein. Interestingly, clusterin mRNA and protein levels were regulated time-dependently by treatment of melanoma cells with 1,25-dihydroxyvitamin D3. Regulation of clusterin mRNA expression by 1,25-dihydroxyvitamin D3 was confirmed by measuring clusterin promoter activity (luciferase assay). Our findings indicate that (i) in contrast to benign acquired melanocytic nevi, clusterin is expressed in primary cutaneous malignant melanomas, metastases of malignant melanoma and melanoma cell lines, (ii) clusterin expression is regulated time-dependently by 1,25-dihydroxyvitamin D3, indicating that antiproliferative effects of 1,25-dihydroxyvitamin D3 on melanoma cell lines may be at least in part mediated via regulation of clusterin expression, (iii) clusterin may be of importance for the growth characteristics of melanoma cells.

P183

Overexpression and hyperphosphorylation of retinoblastoma protein in the progression of malignant melanoma

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Mutation, absence or abnormal functioning of retinoblastoma protein are fundamental elements of uncontrolled growth in human cancer. In this study, we analyze the expression of retinoblastoma protein and phosphorylated retinoblastoma protein in melanocytic tumors in vivo. Real time RT-PCR and immunohistochemistry (tissue microarrays and conventional histological sections) reveal that retinoblastoma protein is progressively upregulated in advanced and metastatic malignant melanomas in vivo. However, this increase is paralleled by increased retinoblastoma protein inactivation due to protein phosphorylation. Interestingly, retinoblastoma protein phosphorylation occurs not homogeneously, but with a "growth zone" related pattern. In superficial spreading melanomas a subepidermal-lateral maximum of phosphorylated retinoblastoma protein can be frequently observed. Accordingly, nodular vertically invasive melanomas are characterized by a strong staining of phosphorylated retinoblastoma protein in deep-dermal invading protrusions of the tumor. Furthermore, Kaplan-Meier analysis of 13 cases of advanced melanomas with long-time follow up suggests a significant negative impact of retinoblastoma protein phosphorylation on survival of melanoma patients independent of tumor thickness. We conclude that the evaluation of phosphorylated retinoblastoma protein in melanocytic tumors could become a helpful adjunct in clinicopathological routine.

P184

Overexpression of c-myb in leukemic and non-leukemic variants of cutaneous T-cell lymphoma

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The c-myb oncogene is a transcription factor that regulates proliferation, differentiation and apoptosis of hematopoietic cells and activated T-cells by binding to promoter sequences of such genes as c-myc or bcl-2 that are expressed in CTCL.
Our study was performed in order to evaluate c-myb expression as a quantitative parameter for differential diagnosis in leukemic and non-leukemic variants of CTCL. C-myb expression was analysed in lesional skin and in the peripheral blood of 21 patients with Mycosis fungoides (MF), 15 patients with Sézary syndrome (SS) and 15 patients with inflammatory skin diseases (ISD) using immunohistochemistry, and semiquantitative as well as quantitative RT-PCR.
Immunohistochemistry confirmed expression of c-myb in the lesional skin of the majority of CTCL patients with a tendency towards higher expression in SS (1.86 ± 0.5) vs. MF (1.2 ± 0.7) while c-myb was absent from the lesional skin of patients with ISD. C-myb was overexpressed in the peripheral blood in all SS patients (100% SS vs. 35,7% MF) at a high expression level (51335.31 $\pm$ 31960.32 AU in SS vs. 1226.35 $\pm$ 1258.29 AU in MF using sqRT-PCR, and 5.72 x 10-2 $\pm$ 2.27 x 10-2 in SS vs. 0.91 x 10-2 $\pm$ 1.18 x 10-2 in MF vs 0.24 x 10-2 $\pm$ 0.11 x 10-2 in ISD using qRT-PCR). CD4+ cells from the peripheral blood of SS patients and cell lines in vitro showed the highest c-myb expression levels upon qRT-PCR (23.27 x 10-2 and 10.78 x 10-2 $\pm$ 7.24 x 10-2).
Overexpression of c-myb in skin lesions of both non-leukemic and leukemic CTCL independent of the stage of the disease indicates that it acts early in disease development. Nevertheless, if positive, c-myb expression in lesional skin is a clear-cut diagnostic marker for CTCL as compered to inflammatory skin diseases. High level expression of c-myb in the peripheral blood as assessed by qRT-PCR constitutes an additional diagnostic parameter for Sézary syndrome and may be especially useful in cases in which morphological determination of Sézary cells or FACS analysis of CD7 and CD26 remain inconclusive.

P185

Selective induction of apoptosis in melanoma cells by a tyrosinase promoter-CD95 ligand construct

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Malignancy of melanoma depends on high chemotherapy resistance, which itself is related to defects in apoptotic signalling cascades and/or increased expression of antiapoptotic proteins. Apoptosis resistance may be overcome by strong overexpression of proapoptotic proteins, as we have shown in a previous study that overexpression of CD95L in human melanoma cell lines activates the CD95/Fas signalling pathway and initiates apoptotic cell death in vitro and in melanoma xenotransplants in mice. However, possible toxic effects of death ligands on nonmalignant cells may limit the therapeutic applicability of this approach. Therefore, selective expression of CD95L in melanoma cells seems to be required. Tyrosinase-derived promoters may be used due to their strong activity restricted to pigment cells.
Luciferase reporter gene assays performed for melanoma cell lines characterized by strong (SK-Mel-19), moderate (SK-Mel-13, MeWo), weak (A-375), and missing expression (M-5) of endogenous tyrosinase revealed high tyrosinase promoter activities in SK-Mel-19, SK-Mel-13 and MeWo, but only weak activities in A-375 and M-5 as well as in nonmelanoma cell lines. Melanoma cell lines investigated by FACS analysis showed significant CD95 surface expression, with the exception of MeWo. Melanoma cells were found highly sensitive to transient transfection of a CMV promoter-CD95L construct as compared to nonmelanoma cells. By applying a tyrosinase promoter-CD95L construct, apoptosis was selectively induced in SK-Mel-19, SK-Mel-13, MeWo as well as in A-375 as clearly shown by DNA fragmentation assays, bisbenzimide staining and analysis of caspase cleavage products. M5 and nonmelanoma cell lines remained uninfluenced. Resistance to agonistic CD95 activation seen in MeWo cells, which are characterized by weak CD95 surface expression, was overcome by overexpression of CD95L.
Our investigations provide evidence that tyrosinase promoter-CD95L constructs selectively induce apoptosis in melanoma cells and may be of value for therapeutic strategies against malignant melanoma.

P186

Mechanism of CD44 shedding in human melanoma cells: Relevance for tumor progression and target for therapeutic intervention

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Previous studies have shown that human melanoma cells (MM) can bind hyaluronic acid (HA), an extracellular matrix component produced by the peritumoral stroma, via the CD44 receptor. Such interaction of HA with membrane-bound CD44 (mCD44) results in the release of autocrine growth factors from MM cells leading to proliferation and progressive tumor growth in vivo. On the other hand, certain MM are capable of shedding soluble CD44 (solCD44) from their surface. Interaction of solCD44 with HA abolishes proliferation of MM in vitro and in vivo by inducing apoptosis. Autocrine or drug induced secretion of solCD44 by MM may thus exert potent anti-tumoral effects.
To elucidate the mechanism of CD44 shedding, a panel of well characterized MM cell lines differing in their capacity to shed solCD44 was analyzed. RNA analysis revealed that solCD44 is not the result of alternative splicing of CD44 mRNAs and the shedding activities are not linked with CD44 mRNA turnover. The differing shedding activities observed are accompanied by similar expression of mCD44 on the cell lines suggesting an increased turnover of the protein. Moreover, CD44-shedding was reduced dramatically by serine protease or metalloprotease inhibitors. Further, mRNA levels of MMP-1,2,3,9 were analyzed by Realtime-RT-PCR. We found the low shedding cell line P2 lacking MMP1 and MMP3 mRNA while the high shedding cell line Bro expressed these MMP. The addition of HA (30 μg/ml) strongly induced MMP1,2,3 expression in the low shedding P2 cell line indicating the regulatory role of the CD44 ligand HA. Since work with other cell types suggested that membrane type MMPs such as MMP14 might be involved in CD44-shedding we analyzed how several established melanoma cell lines differ in their capacity to express MMP14. However, the expression of MMP14 mRNA as well as MMP-14 protein levels on the cell surface did not correlate with CD44 shedding. Our data suggests that another proteases such as ADAM-like proteases might define the observed shedding differences.
These results indicate that MMP14 might not be responsible for differences in CD44 shedding observed among melanoma cell lines. Further, differentially expressed and HA-induced MMPs possibly contribute to CD44-shedding and melanoma progression.

P187

Bone morphogenic proteins are over expressed in malignant melanoma and promote cell invasion and migration.

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Malignant melanoma cells are known to alter their growth factor expression profile compared to normal human melanocytes. These changes probably favor tumor growth and progression, and influence the tumor environment. The induction of TGF-beta 1, -2 and -3 expression in malignant melanoma has been reported before, whereas the expression of related molecules BMPs had not been analyzed in melanomas until today. Here, we show that BMP4 and BMP7 are up-regulated in nine melanoma cell lines, whereas BMP2 is over-expressed in only two of the analyzed cell lines. Immunohistochemistry of primary and metastatic melanoma also showed higher levels of BMP4 and BMP7 expression compared to nevi. Promoter studies revealed that expression was controlled on transcriptional level. The transcription factor ets-1 was identified as a positive regulator for BMP4 regulation. In order to determine the functional relevance of BMP expression in malignant melanoma, chordin expressing cell clones and antisense BMP4 cell clones were generated. The cell clones in which BMP4 activity and expression were reduced showed no changes in proliferation, or in attachment-independent growth when compared to controls. However, a strong reduction of migratory and invasive properties was observed in these cells, suggesting that BMP4 promotes melanoma cell invasion and migration, and, overall, that BMP4 has an important role in progression of malignant melanoma.

P188

Analysis of a truncated, secreted form of P-cadherin in malignant melanoma

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Cadherins are Ca-dependent homophilic cell-cell adhesion molecules which are responsible for correct location of cells and tissue integrity. They are critical for the development and maintenance of epithelial architecture and function, and involve complex cell-cell and cell-substratum interactions. Abberantly expressed cadherins and cadherin expression patterns are known to cause malignant transformation of different types of tissues. In this study, we show the expression of a short 50 kDa form of the N-terminal part of P-cadherin in seven melanoma cell lines compared to melanocytes, keratinocytes and A431. Studies on cell culture supernatant and immunohistochemistry analysis of primary and metastatic melanoma tissue revealed that the short form of P-cadherin is secreted. Analysis on mRNA level revealed only exon 1 to 10 present in the mRNA. However genomic sequence analysis did not show any mutations in melanoma cell lines, neither in the exons or in the canonical AG/GT exon-intron boundaries. There was also no loss of exons 11-16 on the genomic level. We, therefore, hypothesize alternative splicing or genomic translocation to be the reason for this finding. Functionally, the secreted form of P-cadherin could play a role as regulator for the homophilic interaction between P-cadherins by antagonizing their biological role acting as a dominant negative form to interrupt cell attachment.

P189

Endothelial tubulogenesis within fibrin gels is induced by high but not low invasive melanoma cells and requires VEGF

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Angiogenesis is encountered in physiological and pathological situations, for example development, wound healing and tumour induced angiongensesis. Vessel maturation during angiogenesis is characterised by an endothelial cell sprouting from parental vessels, followed by migration and proliferation, alignment and finally the formation of tubular structures. All these processes involve a tightly controlled degradation of extracellular matrix components.
Cell surface receptor-mediated interactions of tumour cells with surrounding structural and cellular components of the stroma as well as soluble factors, released by the tumour cells, are likely to contribute to the activation of stromal cells leading to increased proteolysis of the matrix. However, the molecular mechanisms are is still poorly understood.
The in vivo formation of capillary structures can be mimicked by an in vitro angiognesis assay, in which endothelial cells were cultured on top of a three-dimensional fibrin matrix. By using this in vitro assay, we could demonstrate, that addition of supernatants collected from high but not low invasive melanoma cells induced formation of tubular structures in HDMECs similar to those obtained by incubation of endothelial cells with an angiogenic cocktail. These findings suggest that soluble factors, released from high but not from low invasive melanoma cells are involved in endothelial cell activation. By using a RayBio® human Angiogenesis Array we could show, that VEGF was expressed at high levels in high invasive melanoma cells while it was not expressed in low invasive melanoma cells. Neutralisation of VEGF, using neutralising antibodies, resulted in reduced elongation and migration of HDMECs into the fibrin matrix while anti-bFGF neutralising antibodies failed to inhibit this process. These in vitro findings pointed to VEGF as a major factor which mediates angiogenesis induced by high invasive melanoma cells.

P190

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) efficiently and selectively kills melanoma cells expressing death receptor 4 (DR4)

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent. After binding to one of its two agonistic receptors, DR4 or DR5, it can induce apoptosis in most transformed cells but not or less efficient in normal cells. It has been reported that melanoma cells may be relatively resistant to TRAIL-induced apoptosis and that resistance can be caused by antiapoptotic factors as Bcl-2, Bcl-XL, FLIP and XIAP as well as by downregulation of DR5. In this investigation we addressed the question how TRAIL susceptibility of melanoma cells is correlated with the expression of both receptors.
Sensitivity to TRAIL-induced apoptosis was investigated in 7 melanoma cell lines, of which three (A-375, SK-Mel-13 and Mel-HO) showed significant sensitivity already after 6h, whereas other cell lines showed a moderate increase of apoptosis only after 24 and 48 hours, respectively. Overexpression of Bcl-2 in stably transfected A-375 and Mel-HO clones resulted in a block of the TRAIL sensitivity. Interestingly, DR5 expression was found in all cell lines irrespectively to sensitivity, whereas DR4 was expressed exclusively in highly sensitive cell lines (SK-Mel-13 and A-375), as determined by FACS and Western blot analysis. The third highly sensitive cell line (Mel-HO) was characterized by extraordinary high expression of initiator caspases -8 and -10. There was no correlation between expression of FLIP or XIAP and sensitivity in our panel of melanoma cell lines. Induction of apoptosis by TRAIL resulted in activation of caspase-8 and caspase-3 in the sensitive cells. By employing a specific DR4-blocking antibody apoptosis and caspase activation were prevented in SK-Mel-13 and were significantly reduced in A-375. Apoptosis was less prevented in these cells by an antibody which blocks DR5, whereas this antibody prevented apoptosis completely in MelHO.
These data demonstrate two alternative ways to high TRAIL sensitivity for melanoma cells, either expression of DR4 or high expression of initiatior caspases, whereas there was no correlation between TRAIL sensitivity and DR5 expression, as previously reported. These findings may become of importance for therapeutic strategies against malignant melanoma based on TRAIL.

P191

Dipeptidylpeptidase IV expression modulates the aggressiveness of melanoma cells by differential regulation of MMPs and TIMPs

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The dipeptidylpeptidase IV (DPP IV, CD26) is a multifunctional membrane receptor with an intrinsic enzymatic peptidase activity. The DPP IV gene is expressed in a variety of cell types and its gene product covers functions like interaction with the extracellular matrix, proteolytic cleavage of chemokines as well as the modulation of signaling cascades of other surface receptors. Expression of DPP IV is inversely related with the stage of progression of melanocytic tumors. Pethiyagoda showed, that CD26 inhibits the invasive capacity of melanoma cells (2001). But the exact mechanisms of how CD26 influences the malignancy of the tumor cells is not known.
To address this question, we re-expressed CD26 in a melanoma cell line which does not express CD26 protein neither intracellularly nor on the cell surface as analysed by Westernblot, immunofluorescense microscopy and FACS analysis. CD26 reexpressing cells had a slower growth rate and exhibited a higher level of attachment to fibronectin in Boyden chambers. In gene expression profiling analyses using Affimetrix human U133A_2 chips we could detect a downregulation of MMP-2, TIMP-1 and TIMP-3. TIMP-1 and MMP-2 protein expression was further analysed by ELISA in cell lysates and supernatants. Both, the expression and secretion of TIMP-1 protein was clearly reduced in CD26 expressing melanoma cells in comparison with the MOCK transfected control cells or the non-transfected cell line. We observed a similar reduction in expression of the MMP-2 protein.
Conclusion: Since high levels of TIMP expression can be observed in advanced stages of malignant melanoma and MMP-2 expression correlates with the invasiveness of MM cells, the observed downregulation of MMP-2, TIMP-1 and TIMP-3 is one possible antitumoral mechanism exerted by CD26.

P192

Expression levels of CCR7, CXCR4, CXCL8, MMP2 and MT1-MMP in primary melanomas cannot predict high risk melanomas

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Occurrence of metastases in melanoma patients is fateful and up to date almost unpredictable. Although some factors, correlating with an increased risk for metastases like tumour thickness and ulceration have been identified, little is known about the molecular determinants playing a role in melanoma progression. It would be desirable to estimate the risk of tumour progression by means of further defined factors in any patient and thus to identify high risk patients who might profit from close follow-up and adjuvant therapy.
Recent results demonstrated that several genes are involved in the metastasis of solid tumors. Among these are the chemokine receptors CCR7, CXCR4, the chemokine CXCL8 and the matrix metalloproteinases MMP2 and MT1-MMP. To test whether the expression of these genes correlated with an increased risk of lymph node metastases, we compared the relative expression in primary melanoma from patients with tumour free sentinel lymph node (SLN) negative with those of patients whose SLN were already affected by metastatic spread by means of real time PCR. To this end, 17 patients with negative and 11 patients with positive SLN were analyzed and their relative expression of the respective genes was calculated by the $\Delta\Delta$Ct method. Our data demonstrate an overall high variance in the expression level of the mRNA transcripts investigated. The expression levels of the analyzed genes were generally lower in patients with positive SLN with the exception of CXCL8. In fact, p values were 0.0123 for CXCR4, 0.0403 for MMP2, 0.0058 for MT1-MMP and 0.0619 for CCR7. Notably, expression levels for MMP2 and MT1-MMP which in his active form is necessary for the activation of MMP2 were comparable. In summary, examination of the global expression level of above described genes in primary melanomas seems unsuitable to identify high risk melanomas.

P193

The transcription factor YB-1 is translocated to the nucleus during melanoma progression and is a critical factor in proliferation and chemosensitivity of melanoma cells

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Using PCR-amplified subtractive hybridization of melanocytic nevi and primary melanoma tissues we isolated the DNA-binding protein dbpB/YB-1 overexpressed in primary melanoma. YB-1 is a transcription factor regulating the expression of several genes involved in tumour development and progression. The role of YB-1 in melanoma development is unknown.
To analyse YB-1 protein expression in melanoma progression we generated a polyclonal antiserum against YB-1 and performed semiquantitative western blot analysis and confocal laser scan microscopy of melanoma cell lines and tissues of melanocytic nevi, primary melanoma and melanoma metastases.
The expression analysis of melanocytes and melanoma cell lines which are at different stages of tumour progression indicated that in the course of melanoma progression a translocation of YB-1 from cytoplasm to the nucleus takes place. Melanocytes and radial growth phase (RGP) melanomas showed only a cytoplasmic YB-1 protein expression whereas in vertical growth phase (VGP) melanomas and metastatic melanomas YB-1 is expressed in most cases in the cytoplasm and in the nucleus. In tissues from primary and metastatic melanoma we observed an increased YB-1 protein expression and a translocation to the nucleus. These results indicate that during melanoma progression YB-1 protein is translocated to the nucleus. To reveal the role of YB-1 in melanoma cell proliferation and chemosensitivity we transfected a melanoma cell line with YB-1 siRNA and achieved a stable downregulation of YB-1. The reduced YB-1 expression resulted in a pronounced reduction of melanoma cell proliferation. In addition melanoma cells which have a reduced YB-1 expression show an enhanced resistance against the chemotherapeutic agents cisplatin and etoposide, but not against doxorubicin.
These data highlight that YB-1 has a critical role in melanoma progression and indicate that YB-1 may be a good molecular target in melanoma therapy.

P194

Expression profile of Ezrin/Radixin/Moesin/Merlin in malignant melanoma

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The Ezrin/Radixin/Moesin (ERM) family of actin-binding proteins acts as both: linker between the actin cytoskeleton and plasma membrane proteins and signal transducer in responses involving cytoskeletal remodelling. The neurofibro-matosis type 2 gene-encoded protein merlin is related to the ERM family and therefore the family is now called ERMM. Recent studies suggest that the loss of merlin function contributes to tumor development and metastasis. It is further published that ezrin is a necessary component in the metastasis of osteosarcoma and rhabdomyosarcoma. ERMM are transporter for signals between metastasis-associated cell surface receptor, CD44 that is thought to have a role in the cell migration of invasive tumors and is known to induce intracellular signalling. Additionally, they play an important role in cell adhesion and cell-cell communications. Therefore, we were interested in the expression profile of the ERMM family in the malignant melanoma. We first examined the ERMM expression in 10 different melanoma cell lines in contrast to normal melanocytes and found that Ezrin or merlin is upregulated in melanoma cells in contrast to normal melanocytes, whereas moesin and radixin expression do not differ. Interestingly, merlin was shown to be upregulated in contrast to findings in osteosarcoma. As loss of E-cadherin is an important step in melanoma development we analysed if E-cadherin expression influences ezrin/radixin expression. However, reexpression of E-cadherin in melanoma cells did not result in changes of ezrin/radixin expression compared to the parental cell line.
In summary, ERMM family members are important not only in osteosarcoma, rhabdomyosarcoma and other metastatic tumors but also in malignant melanoma, although their function in melanoma still be analysed in detail.

P195

Are HLA-B8 and HLA-B35 key MHC class I restriction elements for protective T-cell epitopes in melanoma?

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Disease associated misbalances in HLA allele frequencies between the diseased and healthy population may serve as a sign for efficient immune responses, a notion which has been successfully tested for infectious disease. This study is intended to detect misbalances in MHC class I carrier frequencies of advanced melanoma patients compared to healthy bone marrow donors.
HLA-A and B carrier frequencies of 748 stage IV melanoma patients retrieved from serotyping at 6 different centres in Germany were compared to 13,386 fully HLA typed bone marrow donors registered in the German national bone marrow donor registry using a chi-square test.
To this end, the comparison of HLA carrier frequencies in advanced cancer patients with healthy bone marrow donors or patients with loco-regional disease revealed a significant decrease in HLA-A26, HLA-B8 and HLA-B35 carrier frequencies which was also apparent in patients with advanced disease compared to patients with loco-regional disease.
The data suggests that protective immune responses restricted to distinct MHC class I molecules may be operational in a subset of melanoma patients which is the prerequisite for a large scale screen for the corresponding epitopes. The identification of protective epitopes might lead to a significant improvement of the efficacy of the current immunotherapeutic approaches.

P196

Overexpression of BRAF in melanoma cells as an independent promoting factor besides activating BRAF mutations

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The significance of the mitogen-activated protein kinase (MAPK) signaling pathway in melanoma has been supported by the finding of activating BRAF mutations (mainly T1796A/V599E) in about 70% of melanoma samples. BRAF has become a central target in arising strategies for melanoma signaling therapies applying siRNA or kinase inhibitors because of its impact on melanoma progression due to increase of cell proliferation and inhibition of apoptosis.
By applying DNA microarray analysis on 8 melanoma cell lines versus primary melanocytes a panel of up- and down-regulated genes were identified also indicating an increase of BRAF mRNA in melanoma cell lines. Verification by Northern blot analysis revealed a mean upregulation of 3.7-fold at the mRNA level ($p < 0.02$). Quantification of the protein expression by Western blot analysis confirmed with 5.7-fold upregulation ($p < 0,005$) the significant overexpression of BRAF both at the mRNA and protein level.
The melanoma cell lines clustered in two groups, one with moderate overexpression of BRAF at the mRNA level (2-4-fold) and the other with strong overexpression (> 7-fold). 2 of the cell lines with moderate mRNA overexpression showed an additional increase at the protein level suggesting two independent steps of BRAF upregulation.
To identify possible interactions of BRAF overexpression and activating mutations, BRAF exon 15 of the melanoma cell lines was screened by DNA sequence analysis: 6 of the 8 melanoma cell lines showed the respective mutation whereas 2 were WT at 1796. BRAF expression and mutation status did not correlate regarding that one WT cell line showed strong and the other moderate overexpression of BRAF. Proliferation assays, however, revealed that high proliferation rates were associated with high protein overexpression of BRAF independent of the mutation status.
We describe here for the first time a significant BRAF upregulation in melanoma cells suggesting that overexpression and mutation of BRAF are two independent tumor promoting factors in the MAPK pathway of melanoma. These results may be of further impact on novel melanoma signaling therapies.

P197

The serin/threonine kinase casein kinase 1alpha involved in the Wnt/beta-catenin signalling pathway is downregulated during melanoma progression

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The identification of genes specifically overexpressed or repressed in tumour tissues is a key step towards the understanding of the malignant transformation process. Using a PCR-based subtractive hybridization method we compared the gene expression of metastatic and primary melanoma cells isolated from tumour tissues by laser capture microdissection. We identified several genes differentially expressed in metastatic melanoma cells compared to primary melanoma cells. From one isolated cDNA clone (clone 218) we performed expression analysis using RT-PCR and Northern Blot and isolated a full-length cDNA clone by rapid amplification of cDNA-ends (RACE).
RT-PCR analysis of RNA from several different human tissues revealed that clone 218 was expressed in most human tissues. The expression analysis of a panel of melanoma cell lines revealed a decrease of expression of this gene with tumor progression. A reduction of RNA expression about 46% could be observed when radial growth phase melanoma cell lines were compared to metastatic melanoma cell lines and a reduction of 31% compared to vertical growth phase melanoma cell lines. Isolation of a full-length cDNA from melanoma cells and BLAST-search revealed that the isolated cDNA is homologous to casein kinase 1alpha.
Casein kinase 1alpha is a Ser-/Thr-Kinase which is involved in the Wnt-/beta-catenin pathway. Casein kinase 1alpha phosphorylates beta-catenin which subsequently is destined for proteosomal degradation. A reduced casein kinase 1alpha expression leads to a diminished beta-catenin degradation. This might cause an increased cytoplasmic level of free beta-catenin which translocates to the nucleus and acts as a transcription factor in combination with Tcf/LEF-1. Downregulation of casein kinase 1alpha could represent a new mechanism for the increased level of beta-catenin which can be observed in several melanoma samples. This suggests that caseinkinase 1alpha may function as a molecular target for melanoma therapy.

P198

Melanoma inhibitory protein (MIA) influences expression of differential genes

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MIA is a protein produced and secreted by melanoma cells but not by melanocytes. It has been shown that MIA plays an important role in tumor progression in vivo and in regulation of differentiation in vitro. Therefore, we established a cell system in which MIA expression is inhibited via stable antisense MIA cDNA transfection. MIA-deficient cell clones showed changes in cell morphology and re-expressed pigment-relevant genes. Investigating gene expression of the parental melanoma cell line HMB2 compared to MIA-deficient cell clones HMB2-5 and HMB2-8, respectively, in a micro array analysis revealed down regulation of MT1-MMP, tPA, fibronectin, MCAM (MUC18), Cathepsin D, Nestin und Integrin beta 3 (ITGB3) in the MIA-deficient cell clones. Since ITGB3 is known to increase tumor cell invasion, migration, and adhesion we concentrated on ITGB3 expression in melanoma cells. Using this cell system we analyze the role of MIA in regulating ITGB3 expression directly or indirectly as both molecules are involved in tumor development. Further we investigate the role of the transcription factors HMG-1, Snail and ets-1 in ITGB3 regulation as these factors have been shown to be involved in melanoma progression by our group. We, therefore, studied ITGB3 expression in cell clones deficient for HMG-1, ets-1 and Snail and found ITGB3 expression regulated via the transcription factor Snail. Detailed promoter analysis will additionally give further insights into regulation of the ITGB3 promoter.

P199

Loss of expression of proapoptotic Bcl-related proteins Bax and Bak in melanomas with poor prognosis

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At present no therapeutic strategy for metastatic melanoma exists, and a considerably high proportion of patients with primary melanoma will finally develop distal metastases with a 10-year survival prognosis of less than 5%. For patients with primary tumors, prognoses are currently based on statistically confirmed parameters, above all tumor thickness (Breslow index) or depth of tumor invasion (Clark level). Routine histology and immunohistology at present are mainly applied to demarcate melanomas from other skin lesions as well as to define tumor thickness.
Here, a retrospective study was performed on 26 melanoma patients which had been diagnosed with primary tumors of 1.7 - 3.6 mm thickness. After complete excision with safety margins the patients were clinically followed up at minimum for 10 years. Patients were subdivided into two groups: Whereas 12 patients have survived the 10 year follow-up period without recurrance of disease, 14 patients showed tumor progression and died within 10 years (median survival: 25 months). Mean tumor thickness (2.4/2.4 mm) as well as mean age (54/56 years) in both subgroups was highly comparable. In these patients we investigated by immunohistochemistry the expression of proliferation and apoptosis markers, including the antiapoptotic Bcl-2, the proapoptotic proteins Bax, Bak and p53, the proliferation markers Ki-67 and cyclin E as well as the cell cycle inhibitors retinoblastoma protein (RB), p16 (INK4a), p21 (Cip1) and p27(KIP1). Whereas for most markers comparable expression levels were found in both collectives, Bax and Bak showed an increased loss in patients with poor prognosis: Bax was lost in 60% and Bak in 26% versus 17% (Bax) and 0% (Bak) in melanomas from patients with favourable prognosis.
These findings indicate the high potential of molecular markers as prognostic factors for melanoma patients. A systemical survey may finally lead to a consistant pattern of markers, which will allow a more secure prognosis for primary melanoma. Bax and Bak are suitable candidates and their identification as prognostic markers underlines the significance of apoptosis suppression for the progression of malignant melanoma.

P200

Melanoma-associated growth factor/receptor expression in nevi - association with histologic localization?

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Basic fibroblast growth factor (bFGF) and stem cell factor (SCF) belong to the most important growth factors for melanocytes with the receptors FGFR-1 for bFGF and c-kit for SCF. Both factors may be involved in melanoma development as we have shown recently. The dependence of melanocytes on external growth factors is lost in melanoma cells in favor of an autonomous production of many of these factors. We could demonstrate that SCF and c-kit are commonly expressed in primary melanomas similar to the coexpression of bFGF and FGFR-1, the latter has also been reported by other groups.
We were now interested in the expression of growth factors in precursor lesions of melanoma: nevi. Paraffin sections of 25 nondysplastic and 18 dysplastic nevi (25 compound, 13 junctional and 5 dermal) were analyzed for the expression of SCF, c-kit, bFGF and FGFR-1 by the APAAP method.
There was a difference between dysplastic and nondysplastic nevi and between different histological subtypes in regard to the depth of the lesions. Dermal nevi were 100% positive for bFGF and FGFR-1, but 100% negative for SCF and c-kit. In dysplastic junctional nevi, the expression of FGFR-1, SCF and c-kit was increased by 25% compared to nondysplastic junctional nevi, while bFGF was not expressed in both types of lesions. In compound nevi, there was a decreased expression of bFGF, FGFR-1 and SCF and an increased expression of c-kit in dysplastic subtypes compared to nondysplastic subtypes. Over 80% of the papillomatous nevi were highly positive for bFGF and FGFR-1, while 25%-40% expressed SCF and c-kit, respectively. 91% (33/36) of the investigated nevi were positive for c-kit in the epidermis and 83% (24/29) were negative in the dermis.
Taken together, the expression of bFGF was highest in the dermal compartments of nevi, while SCF and c-kit were predominantly found in the epidermal compartments. These data suggest that bFGF serves as a survival factor of nevus cells in the dermis, while in the epidermis the maintained expression of c-kit ensures the responsiveness to the survival factor SCF by the neighboring keratinocytes. With regard to dysplasia, not all growth factors showed an increase in expression, but there seems to be a change in the growth factor expression profile that also depends on the localization of the nevus cells in the skin.

P201

T1796A BRAF mutation is rare in PUVA-associated melanocytic lesions

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Long-term PUVA-treated patients are at risk for melanocytic skin lesions, including possibly cutaneous malignant melanoma (CMM). In particular, PUVA lentigines are present in a high proportion (> 40 to 50%) of patients after long-term PUVA treatment. These lesions frequently display cellular atypia persisting after discontinuance of PUVA treatment. There is concern that PUVA lentigines could be the precursors of melanocytic dysplasia or malignancy. However, the long-term course of (atypical) PUVA lentigines is still unknown and alterations on the molecular level associated with these lesions have not yet been determined. On the other hand, mutations of BRAF (involved in the mitogen-activated protein kinase pathway) have been identified in approximately 40% to 80% of CMM from the normal (non-PUVA-treated) population. Functional studies have also demonstrated an existing T1796A mutation (codon 599) of BRAF being essential for melanoma cell viability and transformation. We therefore investigated whether such BRAF hot spot mutations may also play a role in the formation of PUVA-associated melanocytic lesions, including CMM. Direct DNA sequencing was used to analyze BRAF in DNA extracted from microdissected paraffin-embedded specimens. In total, we analyzed 32 melanocytic lesions, including 15 PUVA lentigines (3 of them with severely atypical features), 12 dysplastic melanocytic nevi, and 5 CMM (2 of them in situ) from a total of 21 long-term PUVA-treated patients. Sequence analysis revealed a hot spot T1796A BRAF mutation in 1 PUVA lentigo and 1 dysplastic nevus, but in none of the 5 CMM. These results suggest that in the major proportion of PUVA-associated melanocytic lesions a BRAF mutation is not required for proliferation and/or transformation into a malignant phenotype. Melanocytic lesions associated with a history of PUVA treatment therefore differ on the molecular level from other melanocytic lesions by the rare occurrence of the T1796A BRAF mutation. Further molecular studies are necessary to determine which other genes are possibly involved in the formation of melanocytic lesions after PUVA treatment.

P202

Expression der zum potentiellen Metastasierungs-Kontroll-Gen nm23-H1 homologen Gene (nm23-H4,-H6 und -H7) im malignen Melanom

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Das maligne Melanom ist aufgrund der ausgeprägten Metastasierungstendenz der bösartigste Tumor der Haut. Die Suche nach einem besseren Verständnis seiner Pathogenese und Chemoresistenzentwicklung sowie nach neuen Therapiekonzepten ist durch die unbefriedigende klinische Situation dieser Tumorpatienten dringend geboten. Ein vorrangiges Ziel der Tumorforschung ist die Identifizierung der Gene, die den metastatischen Phänotyp verursachen oder stabilisieren bzw. unterdrücken
Eines dieser Gene, welches an dem Metastasierungsvorgang einiger Tumoren beteiligt ist, konnte 1988 aus einer Mausmelanomzellinie isoliert werden, das nm23-Gen. Mittlerweile konnte in zahlreichen Studien tumortypabhängig eine Beteiligung an der Tumorprogression bzw. dem Metastasierungsprozeß für nm23-H1 und nm23-H2, das kurze Zeit danach entdeckt wurde, nachgewiesen werden. Zwischenzeitlich wurden weitere homologe Gene (nm23-H3,-H4,-H5,-H6,-H7,-H8) beim Menschen gefunden, über deren Bedeutung für menschliche Tumoren bisher keine Untersuchungen vorliegen.
In einer immunhistochemischen Studie konnten wir eine, im Vergleich zu Nävuszellnävi, starke Immunreaktivität für nm23-H1 in malignen Melanomen und Melanommetastasen nachweisen. Darüber hinaus überprüften wir die Expression der nm23-homologen Gene in Nävuszellnävi, malignen Melanomen und in Melanommetastasen sowie kultivierten Melanozyten und Melanomzellinien mit Hilfe der "real time" PCR und genspezifischen Hybridisierungssonden. Dazu wurde RNA aus kryokonservierten Gewebeproben isoliert, reverse transkribiert und die Expression der Gene nm23-H4, -H6 und -H7 untersucht. Die Ergebnisse zeigten unterschiedlich starke Espressionsstärken sowohl für nm23-H4 also auch nm23-H6 und nm23-H7. Unsere Daten deuten darauf hin dass auch die nm23 homologen Gene ebenso wie nm23-H1 und nm23-H2 eine große Bedeutung bei der Tumorentstehung und Progression des malignen Melanoms besitzen.

P203

Apoptosis induction in melanoma cells by resveratrol does not require hydroxylation to piceatannol and is, in part, independent of caspase-activation and Bcl-2 expression

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Resveratrol and piceatannol are naturally occurring hydroxystilbenes, whose pro-apoptotic activity on some tumor cells has been demonstrated in several experimental systems. Thus, both compounds are interesting antitumoral agents. It has been proposed that the pro-apoptotic activity of resveratrol in some tumors is dependent on its metabolization (hydroxylation) to piceatannol, thus making resveratrol a pro-drug, and that this metabolic step may be catalyzed by the cytochrome P450 isoenzyme, CYP-1B1. Given that the data regarding antitumoral activities of both substances in malignant melanomas, the cutaneous tumor that would most benefit from novel treatment options, are very scant, we have investigated I) whether and how resveratrol and piceatannol induce apoptosis in melanoma cells and II) whether melanoma cells express CYP-1B1 and use this enzyme to metabolize resveratrol into piceatannol.
When four different human melanoma lines (A375, Mel-2a, Mel-HO and MeWo) were treated with either of the two compounds, we could, surprisingly, show by flow cytometry (Annexin V and propidium iodide staining) as well as DNA-fragmentation (Cell Death Detection ELISA) that resveratrol, but not piceatannol, had profound dose-dependent pro-apoptotic activity in the cell lines A375 and Mel-HO, while Mel-2a and MeWo appeared to be rather resistant. Interestingly, inhibition of caspase activity by an oligopeptide pan-caspase inhibitor (zVAD) did not completey abolish resveratrol-induced apoptosis in the two susceptible cell lines. In addition, overexpression of bcl-2 in transfectants of the susceptible lines, A375 and Mel-HO, also did not result in significant reduction of the pro-apoptotic activity of resveratrol. These results suggested that induction of apoptosis by resveratrol is, at least in part, independent of caspase activation and may to some extent overcome bcl-2-dependent resistance against apoptotic stimuli in some melanomas.
When melanoma cells treated with resveratrol or piceatannol were analyzed by mass spectrometry, we found that resveratrol, but not piceatannol, was taken up by the cells from the culture medium. In addition, as detected by Western blot, all melanoma cells tested expressed CYP-1B1 in amounts that did not correlate with susceptibility to resveratrol-induced apoptosis. Since piceatannol could not be detected in resveratrol-treated melanoma cells by mass spectrometry, it appeared that resveratrol was not hydroxylated by these cells, thus strongly suggesting that the apoptotic activity was a direct effect of resveratrol rather than its metabolite, piceatannol.
Overall, our studies shed some unexpected light on the molecular mode of action of resveratrol and suggest that this compound or derivatives with similar activity may be a valuable addition to the currently available armory of antitumoral compounds used for melanoma treatment.

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Invasion of activated melanocytes is stimulated by ultraviolet radiation in human skin reconstructs

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A dysregulation of cutaneous growth factors leading to an uncontrolled proliferation of melanocytes may represent the first step in melanoma development. We have shown recently that an increased expression of bFGF, SCF and ET-3 combined with UVB irradiation can transform melanocytes and lead to melanoma-like lesions in human skin grafted to immunodeficient mice. We now used organotypic skin reconstructs to study the influence of UVA and UVB on melanocytes activated by bFGF, SCF, and ET-3. Prior to the reconstruction of the epidermis, keratinocytes were transduced with the respective growth factors by adenoviral vectors, mixed with the melanocytes at a ratio 3:1 to 5:1 and seeded onto the reconstructed dermis consisting of fibroblasts embedded in collagen type I. At day 3 after air-lifting, skin reconstructs were irradiated with UVA (10-20 J/cm2) or UVB (30-50 mJ/cm2) once or twice. Paraffin sections were analyzed at day 9 by H&E and HMB45 staining. A total of 160 skin reconstructs and 13 different experiments have been performed. An increase in melanocyte numbers within the basal layer by at least 4-fold was observed by bFGF only. The additional exposure to SCF and UVB lead to the development of melanocytic clusters and invasion into the dermis. The latter was also stimulated by UVA, but to a lesser extent. An upward migration of the melanocytes, activated by bFGF and SCF, was seen after irradiation with UVA twice.
Taken together, UV radiation can induce invasion and migration of melanocytes activated by growth factors that are essential for their survival in the skin. As we have shown previously, these effects may have been caused by the UV activation of keratinocytes and fibroblasts via paracrine ways or by UV-induced remodelling of the stroma.

P205

Interference with the serine protease uPA decreases invasiveness of metastatic melanoma cells

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The process of tumor invasion requires several factors including cell adhesion and de-adhesion processes and proteolysis. Proteolytic activity allows tumor cells to degrade the extracellular matrix (ECM) for migration. A key role in tumor cell proteolysis is attributed to the plasminogen activator system, including the serine protease urokinase-type plasminogen activator (uPA), its receptor (uPAR), and the inhibitors PAI-1 and PAI-2. In melanoma, the expression of uPA and uPAR is increased in advanced stages and positively correlates with the invasive potential of melanoma cells. Furthermore, interference with uPA/uPAR expression or function reduced invasion and/or metastasis in animal models of human tumors, including melanoma. For further characterization of the role of the plasminogen activator system in melanoma, we tested the serine protease active site inhibitor WX-UK1 that inhibits uPA. A metastatic melanoma cell line, 1205Lu, was used as a model system. Cell surface uPA activity was assessed by its ability to activate plasminogen. When cells had been treated for 24h with WX-UK1, plasminogen activation was reduced by 50%, compared to its inactive enantiomer as a control. As a next step, activity of WX-UK1 on cell invasiveness was examined in a synthetic ECM-simulating barrier (Matrigel chamber). When cells had been treated with WX-UK1, the amount of invading cells was found to be 10fold decreased. Taken together, WX-UK1 inhibits uPA function in a melanoma cell line. The inhibitor will be used to further establish the role of uPA in melanoma invasion and metastasis and might lead to the development of a new molecular therapeutic strategy for melanoma.

P206

Conditional deletion of VEGF impairs SOS-dependent skin tumour development in transgenic mice

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The vascular endothelial growth factor (VEGF) is a key mediator for normal and abnormal angiogenesis and is implicated in the development of many epidermal tumours. K5-SOS transgenic mice expressing a constitutively active form of the Ras activator Son of Sevenless (SOS) in the basal layers of the epidermis develop skin papillomas at 100% penetrance in a wild-type epidermal growth factor receptor (EGFR) background, whereas in a hypomorphic (wa2) EGFR background tumour development is severely impaired and can be induced by wounding.
To address the role of VEGF during skin tumourigenesis, we generated K5-SOS transgenic mice carrying a conditional (floxed) VEGF allele in an EGFR wild-type (EGFRwa2/+) and hypomorphic (EGFRwa2/wa2) background. Our studies show that deletion of VEGF in basal keratinocytes with a K5-Cre transgenic line severely impairs SOS-dependent skin tumour development. Whereas all EGFRwa2/+ K5-SOS mice develop tumours by 7 weeks of age, 80% of EGFRwa2/+ K5-SOS mice lacking VEGF in the epidermis (VEGFΔep) were still tumour free at this age. Although by 12 weeks, all EGFRwa2/+ K5-SOS VEGFΔep mice had developed tumours, their average tumour volume was significantly smaller than in EGFRwa2/+ K5-SOS mice. The number of blood vessels present in EGFRwa2/+ K5-SOS VEGFΔep skin tumours was significantly reduced suggesting that the delay in SOS-dependent skin tumour development is due to impaired angiogenesis. Surprisingly, in a hypomorphic EGFR background the lack of VEGF expression in keratinocytes completely inhibited SOS-dependent skin tumour development. Even after wounding, EGFRwa2/wa2 K5-SOS VEGFΔep mice remained tumour free for at least 9 months. Interestingly, mice lacking only one VEGF allele started to develop tumours after wounding at the same time as EGFRwa2/wa2 K5-SOS mice, but their tumour volume was significantly smaller. These results show that VEGF deletion in the epidermis severely impairs SOS-dependent tumour growth in a wild-type EGFR background and completely inhibits tumour development in a mutant EGFR background even after wounding. The molecular and cellular mechanisms responsible for these phenotypes will be presented.

P207

Aberrations of chromosome 17p in cutaneous T-cell lymphomas analysed by fluorescence in-situ hybridisation

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Aberrations of chromosome 17p are among the most frequent genomic alterations seen in cutaneous T-cell lymphomas (CTLC). This chromosomal region encodes oncogenes such as p53. We have shown previously that contrasting other tumors, 17p abberrations are not associated with shorter survival in CTCL. In the present study, chromosome 17p was screened for numerical and structural aberrations in 82 samples of 45 patients with CTLC using fluorescence in-situ hybridisation (FISH). The results of cytogenetic studies were correlated with the clinical course of the disease of the patients. A telomeric probe was used for 17p and compared with results from centromeric probes for chromosome 7 and 8. Other candidate tumor genes probes included the regions 7q34-35 (encoding the T-cell receptor beta region) and 8q24 (encoding the myc-oncogen). Minimum and maximum signals of 2-4% of labeled nuclei were regarded as a cut of levels for the probes, respectively. For the chromosome 17p centromeric probe, aberrations were observed in 34 of 82 samples corresponding to 14 out of 45 patients. Comparing these results to CGH screeing all CGH results were confirmed by FISH and, moreover, additional patients were identified. For the chromosome 7 telomeric probes 29 out of 45 patients revealed aberrations of the region 7q34-35, and 8 out 32 patients for 8q24, respectively. When sequential tumor samples obtained from patients during the course of the disease were analysed, a consistent occurence of the aberrations was found. The results of this study demonstrate that FISH analysis of tumor samples of CTLC is a reliable and reproducable technique. The prognostic relevance of the aberrations of chromosomes 7 and 8, but not of chromosome 17p was confirmed.

P208

Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2 independent of ligand binding

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Liver sinusoidal endothelial cells (LSECs) mediate clearance of hyaluronan and scavenger receptor ligands e.g. AGE-modified proteins and oxidized lipids from the circulation. We recently cloned stabilin-1 and -2, two members of a novel family of transmembrane proteins expressed in LSECs. By using primary LSECs and HEK293 cells separately expressing either stabilin, we have investigated their roles in the early events of endocytosis with respect to localisation, ligand binding properties and associations with clathrin and AP-2. Both stabilins were present at the cell surface, although surface levels of stabilin-1 were limited. In addition, stabilins were present in EEA-1+ organelles colocalising with endocytosed AGE-modified albumin. Treating cells with monensin further pronounced this distribution. Recombinant stabilin-2, but not recombinant stabilin-1, bound hyaluronan and the scavenger receptor ligands AGE-modified BSA, formaldehyde-treated BSA and collagen N-terminal pro-peptides. In LSECs both stabilins were associated with clathrin and AP-2, but not with each other. These interactions did not change upon addition of exogenous hyaluronan, suggesting that stabilins are constitutively internalised. In conclusion, hepatic stabilins are both present in the early endocytic pathway, associating with clathrin/AP-2, but whereas stabilin-2- has a clear scavenging profile stabilin-1 does not recognise these ligands.

P209

The human Haarscheibe: a multifunctional nerve end organ in hairy skin.

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The Haarscheibe of human hairy skin, also referred to as touch dome, is considered to be a slowly adapting type 1 mechanoreceptor. Whereas it has been well characterized in hairy skin of cats and rats, only few studies address its presence in human skin. Using dermal and epidermal sheet preparations, we here visualize for the first time its distinct micro-anatomical structure comprising dermal and epidermal elements in situ by confocal laser scanning microscopy. They appeared as dense convolutes of the subepidermal nerve plexus being embedded within distinct, lobulated dermal protrusions clearly demarcated from the surrounding dermal papillae. Corresponding epidermal sheets showed dense aggregations of Merkel cells with a mean number of ~ 140 Merkel cells per Haarscheibe. They extended over 0.217 $\pm$0.130 mm2 and were present at a frequency of 1.457 $\pm$0.549 per cm2. These nerve convolutes contained different fiber qualities, namely Aβ-, Aδ- and C-fibers. Aβ-fibers associate with Merkel cells and account for the mechanosensory properties of Haarscheiben whereas Aδ- and C-fibers are known to play important roles in thermosensation and nociception. Therefore, these findings strongly suggest that the receptive qualities of Haarscheiben exceed mechanosensation and that they may serve as multifunctional nerve end organs in human skin.

P210

Differential Expression of Connexins and Altered Gap Junctional Intercellular Communication Between Melanoma Cells and Cells in its Microenvironment

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Under normal homeostasis, melanocyte growth is controlled by the surrounding keratinocytes through a complex system of paracrine growth factors and cell-cell adhesion molecules. Disrupting this balance can alter the expression of cell-cell adhesion and cell-cell communication molecules and thus provoke the development of melanoma. Gap junctional intercellular communication (GJIC) plays an important role in maintaining tissue homeostasis and is thus a critical factor in the life and death balance of cells. In the skin GJIC is likely to be involved in the regulation of keratinocyte growth, differentiation and migration as well as in keratinocyte-melanocyte interactions. Ten of the 21 connexins are expressed in human skin. Changes in connexin expression, in particular loss of Connexin (Cx) 43, may result in a reduction or a loss of gap junctional activity, which is thought to contribute to tumor progression.
Using microarrays and RT-PCR, we show that Cx43 is not or only weakly expressed in melanoma cells, but highly in melanocytes, keratinocytes, fibroblasts and endothelial cells. On the other hand, Cx32 is expressed in melanoma cells and, at a lower level, in melanocytes, but not in keratinocytes, fibroblasts and endothelial cells. Cx26 and Cx30 are only weakly expressed in a minor portion of melanoma lines. Using immunofluorescence microscopy on human tissue sections, we did not detect Cx43, Cx26 and Cx30 in melanoma. Interestingly, we show an induction of Cx26 and Cx30 in the epidermis adjacent to melanoma, while both are not detectable in the epidermis adjacent to melanocytic nevi. Using specific markers (cytokeratin 6 (CK6) and 17 (CK17) respectively), we show that the induction of Cx26 and Cx30 is not a reflection of reactive hyperproliferative or traumatized epidermis. Dye transfer experiments with cultured cells show that - in contrast to the highly communicating keratinocytes - there is GJIC only between subpopulations of melanocytes and also melanoma cells. In some cases there appears to be unidirectional communication between melanoma cells and fibroblasts. RNAi for connexins using lentiviral vectors will determine the putative roles of GJIC in tumor progression.

P211

A murine model for human leukocyte-adhesion deficiency-1 wound-healing deficiency due to impaired myofibroblast differentiation resulting from reduced TGF-β1 signaling

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Wound healing is a highly dynamic process relying on the participation of resident and blood cells. Active migration of leukocytes into the wound is a prerequisite. Disruption of leukocyte migration occurs in leukocyte-adhesion deficiency 1 (LAD1), and leads to severely impaired wound healing. In order to study the underlying mechanisms, we employed a murine model for LAD1. We found that sizes of cutaneous full-thickness wounds in CD18-/- mice were highly increased during the phase of wound contraction between days 5 and 7 which is mainly controlled by myofibroblasts. We therefore studied the expression of key markers in myofibroblast differentiation. Both ED-A fibronectin and α-smooth muscle actin were substantially reduced in CD18-/- mice, indicating defective myofibroblast differentiation. TGF-β1 and its receptor TGF-βRII were also largely decreased. Since TGF-β1 is a key factor promoting wound contraction, we supplemented TGF-β1 by repeated injections into wound margins. This fully rescued wound closure in CD18-/- mice. Since in wounds of CD18-/- mice defective migration leads to a severe reduction of neutrophils, we envisioned that infiltrating macrophages may not be able to phagocytose apoptotic CD18-/- neutrophils, thus, lacking their main stimulus to secrete TGF-β1. We demonstrated that in the absence of neutrophils, or in co-cultures with CD18-/- neutrophils, TGF-β1 release by macrophages was dramatically reduced due to defective phagocytic clearance of CD18-/- neutrophils, whereas pro-inflammatory cytokines were increased. Deviant from former views, our data consistently demonstrate that paracrine secretion of growth factors induced by wound-infiltrating neutrophils is essential for cellular differentiation in wound healing.

P212

cFLIPL interferes with Receptor-interacting Protein (RIP) recruitment and its posttranslational modification at the TRAIL death-inducing signalling complex (DISC) in human keratinocytes

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Human keratinocytes undergo apoptosis following treatment with TNF-related apoptosis-inducing ligand (TRAIL). TRAIL also triggers non-apoptotic signalling pathways including activation of the transcription factor NF-κB. The intracellular protein cFLIPL is a potent inhibitor of death receptor-mediated apoptosis. Functional analysis with differentially cFLIPL-expressing keratinocytes revealed that cFLIPL blocks TRAIL-mediated apoptosis, NF-κB activation as well as expression of the NF-κB target gene interleukin 8 (IL-8) at the death-inducing signalling complex (DISC). Surprisingly, cFLIPL did not significantly impair the enzymatic activity of the receptor complex as determined by its ability to cleave the prototypical caspase 8 substrate Bid in an in vitro cleavage assay. In contrast, DISC analysis revealed recruitment of receptor-interacting protein (RIP) to the TRAIL DISC in keratinocytes. This recruitment and the detectable posttranslational modification of RIP was abrogated in cFLIPL-overexpressing cells. Studies using the proteasomal inhibitor MG115 indicated that these posttranslational modifications represent ubiquitinylated forms of RIP generated within the TRAIL DISC, because they were undetectable in total cellular lysates.
Taken together, our data demonstrate that cFLIPL is not only a central anti-apoptotic modulator of TRAIL-mediated apoptosis, but also an inhibitor of TRAIL-induced NF-κB activation. Moreover, we have identified cFLIPL as an inhibitor of RIP recruitment and posttranslational modification of this molecule in the TRAIL DISC. These modifications of RIP may be critically required for TRAIL-dependent NF-κB activation and subsequent pro-inflammatory target gene expression. Hence cFLIPL modulation in keratinocytes does not only influence apoptosis sensitivity but may also modify non-apoptotic death receptor signalling crucial for distinct proinflammatory responses in the skin.

P213

Increased adhesion of peripheral neutrophils to human Thy-1 (CD90) in psoriasis - establishment of an in vitro adhesion model for therapeutic and diagnostic investigations

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The chronic inflammatory skin disease psoriasis is characterized by a prominent infiltration and microabscess formation by neutrophils. Recent studies have emphasized that the overexpression of endothelial cell adhesion molecules, regulating the extravasation of leukocytes into perivascular tissue is a crucial event in the development of psoriasis. Since the human Thy-1 (CD90) is an activation-associated endothelial cell (EC) adhesion molecule involved in the adhesion of neutrophils to activated endothelium via the interaction with the β2-leukocyte integrin Mac-1 (CD11b/CD18), the role of Thy-1-mediated neutrophil adhesion in psoriasis was investigated.
The adhesion of peripheral blood neutrophils of patients suffering from psoriasis vulgaris to Thy-1-transfected cells as well as to activated Thy-1-expressing human dermal microvascular EC is conspicuously increased in comparison with the adhesion of neutrophils from healthy controls. Furthermore, the adhesion of peripheral blood neutrophils to Thy-1 is correlated to the activity of the disease (PASI). In contrast, psoriatic and healthy neutrophils adhered in a similar extent to ICAM-1-transfected cells. Moreover, soluble mediators released by psoriatic mononuclear cells were able to stimulate the Thy-1-mediated adhesion of neutrophils, whereas supernatants of healthy MNC did not show any effect suggesting an inflammation-boosting effect of MNC-activation in psoriasis. The distinct reduction of this adhesion by blocking TNFalpha or IL-8 showed the critical role of these cytokines in the adhesion-boosting effect of MNC-activation.
In conclusion, the Thy-1-mediated adhesion seems to be a very important adhesion mechanism to realize the attachment of neutrophils to activated endothelium in physiological processes as well as in chronic inflammatory diseases.

P214

Keratinocytes express N-methyl-D-aspartate-receptors of the NMDAR2D-type

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N-methyl-D-aspartate-receptors (NMDA-receptors) are ionotropic glutamate receptors (ligand-gated ion-channel proteins), which can elevate the intracellular calcium concentration of keratinocytes. The receptor consists of a constant NR1 subunit combined with at least one type of NR2 subunits (NR2A-D). The identification of the NR2 subunit is important in determining both biophysical and pharmacological properties of the receptor. The present study was therefore performed to identify the NR2 subunit of NMDA-receptors in keratinocytes.
The expression of NR1 and NR2 A-D was investigated in HaCaT-cells and native human epidermal keratinocytes (NHEK) by RT-PCR. Because it is known that the subtypes of NMDA-receptors have different physical properties, additional functional tests were performed using the selective agonist N-methyl-D-aspartate (NMDA) in HaCaT-cells. Herein the intracellular calcium concentration was studied in standard-HBS and magnesium-free and glycin-containing medias.
NR1 could be detected in both HaCaT-cells and NHEK by RT-PCR. Both cell lines also express the NR2D-subunit, while NR2A-C were not detected. Compared to Mg-containing and glycin-free media (standard-HBS) there was a reduced number of cells with elevated intracellular calcium concentration using magnesium-free and glycin-containing medias.
The NMDA-receptor of keratinocytes seems to be of the NMDAR2D-subtype. The low sensitivity of cultured keratinocytes to magnesium and glycin corresponds well to experiences for NMDAR2D in neuronal cells. Further studies should proof the possible therapeutic impact of this observation.

P215

Dendritic cells (DC) interact specifically with dermal fibroblast via the Thy-1/Mac-1 adhesion system: Regulation during DC maturation and consequences for DC migration

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To trigger an effective T-cell-mediated immune responses DC have to migrate from peripheral tissues such as the epidermis, via the dermis into locally draining lymph nodes (LN) where they present antigen to naive T cells, During migration DC undergo distinct phenotypic and functional changes termed collectively DC maturation, which can be recapitulated during in vitro DC culture. To date little is known about the interactions with the various cellular microenvironments that DC encounter during their migration from peripheral epithelia to lymphoid tissues.
Here, we report for the first time that human DC have the capacity to interact specifically with human dermal fibroblasts (HDF). First, in psoriatic skin a close apposition marked co-localization of CD1a+ DC and Thy-1+ HDF was shown. In vitro, DC were generated from CD14+ PBMC using GM-CSF and IL-4 containing media. Immature day 5 DC adhered strongly to HDF. Blocking Thy-1 on HDF or Mac-1 on DC resulted in significantly reduced adhesion up to 60% identifying the Thy1/Mac1 adhesion system as an important mechanism for this interaction. These findings are underlined by a strong adhesion of DC to Thy-1-transfected CHO-cells, whereas almost no binding to vector-transfected control cells could be observed.
Preliminary results suggest that Thy-1-stimulation of DC results in distinct signaling events and the induction of downstream genes and cellular motility as shown by cDNA microarrays and the analysis of cell trajectories by time-lapse video microscopy.
Interestingly, the capacity of mature day 6 DC to adhere to HDF as well as to Thy-1-CHO was markedly reduced compared to immature day 5 DC. Correspondingly, day 6 mature DC downregulated their surface expression of the β2-intergin Mac-1.
Our data indicate that during maturation DC down-regulate adhesion mechanisms such as the Mac-1/Thy-1 pair mediating their interaction with HDF, which in turn enhances their capacity to migrate to the draining LN. These data are consistent with the notion that dermal fibroblasts influence the migratory capacity of DC and may thus play an active role in the regulation of DC immune functions.

P216

Expression of the human proteinase inhibitor hurpin (serpinB13) in transgenic mice: abnormal hair development and resistance to UV-induced apoptosis

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Hurpin is an intracellular, differentially spliced member of the serpin superfamily which is downregulated by UVB light and was initially found to be overexpressed in psoriatic skin. Keratinocytes were shown to be protected from UVB-induced apoptosis when overexpressing hurpin. Therefore, hurpin is believed to be involved in cellular processes including differentiation and apoptosis. It has been shown that hurpin selectively inhibits the lysosomal cysteine proteinase Cathepsin L. This proteinase has been linked to quite different functions including epidermal homeostasis, regulation of the hair cycle, MHC class II-mediated antigen presentation and NKT cell development. Interestingly, Cathepsin L was frequently found to be overexpressed in squamous cell carcinoma. Using newly generated monoclonal antibodies against hurpin we found that, in addition to being upregulated in psoriatic skin, hurpin is overexpressed and redistributed in inflammatory dermatoses, benign and malignant tumors.

To further study the physiological role of hurpin, we generated transgenic mice expressing the human hurpin using a 80 kb fragment of genomic DNA. By RT-PCR analysis we found hurpin expression to be restricted to the skin of transgenic animals. At the age of about eight weeks, we observed noticeable abnormalities (hair loss/shortening) regarding the abdominal hair, only. Further studies showed differences in the staging of the hair cycle between wild type and transgenic animals. To further characterize the hurpin-transgenic mice, we irradiated transgenic and wild type mice using UVB-light. Subsequently, we analysed the appearance of apoptotic cells in skin sections. Interestingly, the number of apoptotic cells was markedly reduced in transgenic mice.
Our results suggest that hurpin is involved in numerous processes in the skin which might be correlated to the inhibition of Cathepsin L but possibly include Cathepsin L-independent functions. Further studies will elucidate the pathophysiological role of hurpin.

P217

TRAIL induces activation of different mitogen-activated protein kinases (MAPK) in human keratinocytes

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Keratinocytes activate an apoptotic program following ligation of surface molecules including TNF-related apoptosis-inducing ligand (TRAIL) receptors. Recent evidence indicates that beside caspase-mediated apoptotic signals, these receptors may also activate non-apoptotic signalling pathways like the transcription factor NF-κB or mitogen-activated protein kinases (MAPK). MAPK of major relevance in human keratinocytes include the extracellular-signal-related kinases 1 und 2 (ERK1/2), c-Jun N-terminal kinases 1, 2 (JNK1, 2) and p38 with its isoforms α, β and δ.
To investigate if TRAIL activates MAPK, we analyzed posttranslational MAPK modifications following TRAIL stimulation in human keratinocytes. Phosphorylated active forms of the MAPK ERK1/2 and p38 were rapidly induced within minutes, while JNK activation was marginal. This MAPK activation was independent of active caspases, because neither ERK nor p38 activation were inhibited by the pancaspase inhibitor zVAD-fmk. In order to block caspases more physiologically, keratinocytes were stably transfected with the intracellular caspase-8 inhibitor cFLIP(L) and studied for MAPK activation. Interestingly, TRAIL leads to ERK activation irrespective of cFLIP(L) expression. We next investigated TRAIL-induced interleukin (IL)-8 secretion in the presence of different MAPK inhibitors. However, although ERK inhibitor U0126 completely blocked TRAIL-induced ERK activation, it did not alter TRAIL-dependent IL-8 secretion. Further studies examining the impact of TRAIL-induced p38 activation for IL-8 expression are currently underway.
We conclude that TRAIL activates not only apoptosis and NF-κB, but also different MAPK in human keratinocytes. TRAIL-induced MAPK activation might thereby initiate apoptosis-independent signals including differentiation, proliferation and inflammation. We speculate that this signalling pathway might be particularly important in TRAIL-resistant keratinocytes expressing high levels of cFLIP(L), thereby using TRAIL as a potentially hazardous growth stimulus.

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Selective upregulation of murine ß-defensin 3 after disruption of the permeability barrier - specific role in defense against Pseudomonas aeruginosa

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Protection of the skin against microbiological infection is provided by the physical permeability barrier and by antimicrobial proteins of the innate immune system including ß-defensins. Injury of the physical barrier is a prerequisite for experimental bacterial infection. We asked whether the expression of murine ß-defensins (mBDs) is stimulated in vitro and by disruption of the permeability barrier or by contact with bacterial supernatant in vivo. First, our sequence analysis revealed that mBD-1, -3 and -14 are homologous to the well-characterized human ß-defensins hBD-1, -2 and -3, respectively. mßD-1 and -14 were found to be expressed constitutively in embryonic and 5 days old mice. mBD-3 was not expressed constitutively, but induced in adult skin after contact with environmental germs. Disruption of the permeability barrier by tape-stripping led to a significant up-regulation of mBD-3 expression, only. A further pronounced stimulation of mBD-3 occurred after stimulation with Pseudomonas aeruginosa supernatant. In contrast, mBD-3 was only slightly stimulated by Staphylococcus aureus supernatant. However, Staphylococcus aureus significantly stimulated mBD-14 expression. These results show that mouse epidermis contains constitutively expressed and stimulated defensins. mBD-3 is the only defensin upregulated by permeability barrier disruption. The injury of the skin causes a loss of body fluid, which lead to a wet wound a prerequisite for Pseudomonas aeruginosa colonization. Therefore, our results suggest that the selective upregulation of mBD-3 after skin barrier disruption is primarily a defense against gram negative bacteria colonizing like Pseudomonas aeruginosa, a well-known problem in open wet wounds like leg ulcers.

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Interleukin-1 mediated enhancement of UVB-induced apoptosis is NFκBdependent||| ABSATZ||| E. Strozyk [1], B. Pöppelmann[1], T. Schwarz [2], D. Kulms [3]
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The pro-inflammatory cytokine interleukin (IL)-1 can markedly influence apoptosis induced by different stimuli. This phenomenon affects not only death receptor-mediated apoptosis, induced by FasL or TRAIL, but also UVB-induced apoptosis, a much more complex process which involves DNA damage, ligand independent death receptor activation and generation of reactive oxygen species. IL-1 is an activator of the transcription factor NFκB. IL-1 receptor activation leads to induction of a kinase cascade, resulting in phosphorylation of the cellular inhibitor of NFκB (IκB) and its subsequent proteasomal degradation, followed by NFκB liberation and nuclear translocation. There NFκB acts as potent transcription factor. Activation of NFκB is generally regarded to be associated with the induction of anti-apoptotic pathways, causing up-regulation of several anti-apoptotic genes like IAPs and FLIP. Consequently, death receptor-mediated apoptosis is reduced in the presence of IL-1. In contrast, apoptosis caused by UVB is enhanced by IL-1 which coincides with a strong release of the pro-apoptotic cytokine tumor necrosis factor alpha (TNFα). The involvement of NFκB in the regulation of TNFα release and modulation of apoptosis was confirmed by use of the IκB kinase (IKK) inhibitor BAY11-7082 and the proteasome inhibitors MG-132 and lactacystin. Immobilisation of NFκB in complex with IκB resulted in reversion of the IL-1 effect on both death ligand- and UVB-induced apoptosis. Accordingly, BAY11-7082 , MG-132 and lactacystin inhibited TNFα release induced by the combination of IL-1+UVB. This type of selective and differential NFκB-mediated gene expression seems to be a feature specific for genes involved in manipulation of apoptosis, since expression of another IL-1/ NFκB-inducible gene encoding the pro-inflammatory cytokine IL-6 remained unaffected by any pro-apoptotic co-stimulus. Taken together, these data suggest different control mechanisms to be responsible for IL-1-induced and NFκB-mediated cytokine release, depending on its physiological role causing either inflammation or apoptosis.

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Is corticotropin-releasing hormone (CRH) a maturation factor for human skin mast cells?

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It has previously been reported that the key stress response-hormone, corticotropin-releasing hormone (CRH), for which human skin and hair follicles (HF) are now recognized as major extra-hypothalamic expression sites (Ito et al. JID 2004, Slominski et al. Endocrinology 2004), can activate skin mast cells (MC). Here we report preliminary evidence that CRH may even stimulate the maturation of mast cells from resident, immature precursors. The number of c-kit-positive cells is much higher than that of Giemsa- positive cells, suggesting that human hair follicle (HF) pools immature MCs in the HF connective tissue sheath (CTS). If human scalp hair follicles (HF) in anagen VI are microdissected and organ-cultured for several days, one notices emigration of cells with morphological/histochemical MC characteristics from the HF connective tissue sheath, which is now considered a major source of mesenchymal stem cells in the skin. The number of emigrating MC-like cells is upregulated if CRH is added to the culture. CRH addition also not only upregulates the percentage of degranulated MC within the CTS, but also the total number of histochemically detectable CTS-MC (as assessed by Giemsa histochemistry). Since we found the major cutaneous CRH receptor (CRH-R1) to be abundantly expressed in human HF epithelium, but not in the CTS c-kit-positive cells, we then checked whether CRH up-regulates the expression of stem cell factor (SCF), a major MC differentiation factor. CRH incubation of human scalp HFs does indeed stimulate intraepithelial SCF immunoreactivity, and hair follicle steady state-levels for SCF mRNA. Therefore, CRH may affect CTS-MCs and their precursors indirectly, e.g. via stimulation of SCF release from the HF epithelium. We are currently exploring whether co-culture of HFs with CRH and SCF-neutralizing antibody abrogates at least in part the CRH-induced effects on CTS-MC summarized above. Our currently available data suggest that CRH, indirectly via stimulation of SCF release from the HF epithelium, stimulates immature human CTS-MC precursors (or even mesenchymal stem cells in the CTS?) to differentiate into mature skin MC in vitro. This points to previously unknown neuroendocrine controls of human skin MC biology in situ, and a key role for CRH in these controls.

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Staphylococcus aureus Modulates Expression of Epidermal Tight Junction-Proteins

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A pivotal function of the skin is to prevent invasions of microbial pathogens. Although the impact of various bacteria and their virulence factors on proteins comprising the tight junction (TJ) and zonula adherens have been investigated in simple epithelia of intestine, there is no data available about the regulation of the TJ and/or the zonula adherens during skin infection. Therefore, we investigated the influence of the pathogen S. aureus and the commensal S. epidermidis on the expression of epidermal TJ, zonula adherens and the attached actin cytoskeleton. We established a porcine skin infection model: punch biopsies were infected for 24 hours with S. aureus (ATTC 29213 and a clinical wild-type isolate) and S. epidermidis (1457) being in the postexponential phase. Subsequently, expression of the TJ proteins claudin 1, ZO-1 and occludin as well as the zonula adherens proteins E-Cadherin and β-catenin plus the actin cytoskeleton were determined by immunohistochemical stainings. We found, that compared to the control group, S. aureus induced down regulation of claudin 1, ZO-1 and the zonula adhaerens, whereas S. epidermidis exerted no such effects. With respect to the expression of occludin and actin we observed no alterations after staphylococcus infection. Ki67/TUNEL stainings revealed non significant differences of apoptosis and proliferation within the infected compared to control skin, indicating skin vitality.
In summary we demonstrate for the first time that S. aureus induces down-regulation of TJ and zonula adherens proteins within the skin and give further insight into to the yet unrecognized mechanisms how dermal infections may alter TJ morphology.

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ET-1 induction of collagen synthesis in fibroblasts requires distinct G-protein signaling cascades

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ET-1 initiates a series of signaling events resulting in various cellular responses. ET-1#s role in tissue remodeling and development of fibrosis had been demonstrated in multiple in vitro models to include protein kinase C (PKC) as a pivotal intracellular switch. Using cultured human dermal fibroblasts, we aimed to identify the cell surface receptor responsible for the signal transduction of the ET-1 inducible collagen type I (coII) synthesis and the signaling pathway connecting the receptor and PKC. The signaling of ET-1 is generally mediated by two receptor subtypes, the ETA and ETB. The receptors transduce their stimulation to the intracellular compartment via G-proteins, whereby the ETA couples to Gs, Gq and in some cell types to Gi, whereas the ETB couples to Gq and Gi.
Initially, we analysed the expression pattern of ET recptors. In contrast to the ETB, the ETA was clearly detectable by immunoblotting. Cell fractionation revealed that the ETA was mainly localised within the cell membrane fraction. Using a 125I-ET-1 displacement assay with specific antagonists against the ETA (BQ123) and the ETB (BQ788), expression of the ETA was confirmed. The IC50 values of ET-1 and PD156252 were in accordance to published data. The IC50 value of BQ123 however was increased by a factor of ten. ET-1 stimulation of the cells increased the amount of coll by a factor of two compared to unstimulated controls.
To determine the type of G-protein(s) responsible for the effect of ET-1 on coll synthesis, specific inhibitors were applied. Pertussis toxin (Ptx), a Gi inhibitor, had no influence on the basal coll synthesis but strongly decreased ET-1-induced coll synthesis. The Gs was rendered unstimulable by preincubation of the cells with cholera toxin and revealed results comparable to the Ptx experiments. Finally the downstream effector of the Gq, PLC, was inhibited by a specific inhibitor U73122. This approach did not influence ET-1-induced coll synthesis.
These data indicate that ET-1 stimulated coll synthesis is probably mediated via the Gi and/or Gs and not due to activation of Gq and its subsequent activation of PLC. Further analysis will focus on downstream effectors of the Gi and Gs to clarify the role of intracellular pathways stimulating PKC which obviate the Gq coupled PLCb signaling. These results may help understand the molecular mechanisms underlying in fibrotic processes.

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High PAI-2 expression as a hallmark of scleroderma fibroblasts in vitro

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Systemic scleroderma is a chronic disease, which leads to fibrosis of the skin and internal organs. Fibroblasts obtained from lesional skin of patients with this disease exhibit an activated state when cultured. Activation includes enhanced production of collagen I and other matrix molecules, increased release of TGF-b, CTGF, MCP-1 or PN-1, but no reliable highly expressed markers have so far been identified.
In this study, we report strong (>20fold) constitutive overexpression of PAI-2 in scleroderma fibroblasts compared to controls. Some, but not all patient strains demonstrated enhanced (no more than 2fold) collagen production. Induced PAI-2 expression was observed at the RNA and protein level. Immunolocalization showed PAI-2 signals in 60% of scleroderma and 20% of control fibroblasts in a non-uniform expression pattern. Induction persisted over 12 passages in culture and was strictly dependent on presence of serum (10% FCS). Presence of TNF-a, a known potent inducer of PAI-2, was not detected, and ELISA assays showed that all sera added contained negligible amounts of TNF-a below assay detection limits. CTGF and TGF-b, potentially present at elevated levels in patient cell cultures, were also ruled out as agents stimulating PAI-2 production.
PAI-2 belongs to the serpin supergene family, presumed to participate in regulation of plasmin production. Interestingly, PN-1, which also inhibits plasmin production, was found elevated in scleroderma. Thereby both, PAI-2 and PN-1, directly or indirectly inhibit MMP activity and can in this way contribute to connective tissue accumulation and breakdown.
Interestingly, we did not detect any significant PAI-2 levels secreted into the medium, and high levels were only seen intracellularly. Its function inside cells is unclear at present, but may include control of cell survival.
We describe PAI-2 as a novel marker of cultured scleroderma fibroblasts, which is expressed at more than 20fold higher levels than in control cells. Induction depends on an unidentified stimulator in FCS, which is not TNF-a, TGF-b or CTGF, and is restricted to the intracellular compartment.

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Lack of mechanical stimulation results in a "pro-inflammatory" fibroblast phenotype

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Fibroblasts are continuously subjected to changes in mechanical forces and are versatile to adapt to an increase or a decrease of tensile stress. To better understand the changes in gene expression in response to these forces, we cultured skin fibroblasts in collagen lattices that were either relaxed, i.e. not stressed, or mechanically stressed. The transcriptional profile was assessed by cDNA microarray analysis when the contraction process of relaxed lattices was macroscopically terminated (20 hours).
Absence of stress resulted in a lattice diameter of about one tenth of the initial area, brought about by the isotonic contraction of fibroblasts. The cells had assumed a rounded morphology and lacked a-smooth muscle-positive stress fibers and vinculin-positive focal adhesions. The expression profile revealed upregulation of genes involved in apoptosis, of inflammatory mediators such as IL-1, IL-6, COX-2 and ICAM-1, and of numerous proteases including MMPs.
COX-2 (cyclooxygenase-2) expression was examined in more detail, because it was induced to the highest extent (20fold in comparison to stressed lattices) in the entire screen. COX-2 has been described as an inducible pro-inflammatory enzyme catalyzing the initial steps in prostaglandin synthesis. Fibroblasts in relaxed lattices showed high COX-2 expression at 12 hours. Surprisingly, at earlier time points (0.5 - 8 hours), expression was increased in stressed fibroblasts. It appears that COX-2 is tightly regulated in a bi-phasic manner.
The pattern of IL-1b expression was strikingly similar to that of COX-2. IL-6 was also strongly induced in relaxed fibroblasts after 12 hours, but no signals were detected at any time in stressed lattices. Blocking IL-1 signaling prevented induction of IL-6 and COX-2 but not that of MMP-1 or KGF.
These results suggest a hierarchical activation of pro-inflammatory genes in fibroblasts not exposed to mechanical forces. We propose that such fibroblasts assume a "pro-inflammatory" phenotype turning them into active key players in tissue inflammation.

P226

Enrichment of stem cells from human native epidermal keratinocytes

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Due to their proliferative potential, epidermal stem cells have a special importance for the therapeutical use in tissue engineering and as a target for investigations of drug effects, e.g. in the therapy of hyperproliferative skin diseases such as psoriasis.
Some published methods of stem cell isolation from keratinocytes of primary cultures or excised skin use the different adhesion behavior on extracellular matrix proteins. Thereby stem cells adhere faster than transit amplifying cells (TA cells) and suprabasal cells. For our investigations, we used a mixture of bovine collagen I (30 μg/ml) and human fibronectin (10 μg/ml) as well as human collagen IV (50 μg/ml). In comparison to other studies, the adhesion times were modified from 10 to 5 min for collagen-I-fibronectin and from 20 to 5 min for collagen IV, in order to ensure the predominant adherence of stem cells. After the isolation procedure, the total cell population, the supernatant and adhered cells were characterized by flow cytometry using the surface marker ß1-integrin. In this context, the adhered cells showed an exceptionally high expression of ß1-integrin, very typical for stem cells. With the combination of α6-integrin or ß1-integrin and the transferrin receptor, the stem cells should be differentiable from the TA cells. However, in contrast to the literature, in this study the adhered cells also exhibited a definitely positive expression of the transferrin receptor. It remains to be elucidate, if the use of different anti transferrin-receptor antibodies affected these results (other studies used a self-developed antibody; present study used commercial antibodies).

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Mechanisms of Internalization and Recycling of Somatostatin Receptor-1

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In the skin, somatostatin (SST) immunoreactivity has been demonstrated in nerve fibers, keratinocytes, Merkel cells, and dendritic cells. SST is described as a major inhibitor of exocrine and endocrine secretion and analogs of SST have been found to inhibit tumor proliferation. The effect of SST is mediated by five somatostatin-receptor subtypes. Stimulation with SST induce endocytosis of the somatostatin-receptor subtypes, thus it is important to understand the mechanism and function of receptor-subtype specific trafficking. Somatostatin-14 (SST-14) is rapidly and efficiently internalized by somatostatin receptor 1. We quantified somatostatin receptor 1 mediated endocytosis of 125I-Tyr11-SST-14 by cell surface binding assays and uptake assay. The fate of internalized SST-14 was characterized by HPLC analysis of endocytosed ligand. Internalized FITC-SST-14 and biotinylated sst-1 was localized by confocal microscopy. Somatostatin receptor 1 mediated a fast and dynamic process of endocytosis, recycling, and re-endocytosis of somatostatin-14. Ligand uptake assays and quantification of cell surface binding sites demonstrated that during the first 2 minutes of stimulation approximately 50% of ligand occupied sst-1 internalized. Somatostatin receptor 1 mediated an uptake of SST-14 into acidic wash resistant cell compartments. Chronic stimulation with SST-14 induced accumulation of approximately 2.2 times the amount of cell surface bound SST-14 into the cells. The ratio between cell surface bound SST-14 and cell associated SST-14 is independent from the concentration of the ligand used to stimulate the cells. Endocytosed SST-14 recycles somatostatin receptor 1 mediated into the medium. After the extra cellular localized ligand is removed approximately 20-25% of cell-associated SST-14 could be detected into the surrounding medium during the 1st min of incubation. SST-14 is slowly degraded by phosphoramidon sensitive endopeptidases. Thus, the fast somatostatin-receptor 1 mediated recycling of biologically active SST-14 suggests a biological function of the somatostatin receptor 1 as an autoreceptor.

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Important Role of Rab proteins on the Regulation of Neurokinin-1 Receptor Trafficking

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Neurokinins such as substance P (SP) play a pivotal role in neurogenic inflammation, pruritus and pain. SP is released from unmyelinated sensory nerve fibers of the skin thereby causing erythema, wealing, itching and immunomodulation. Neurokinins exert their effects by binding and activating neurokinin receptors. SP induces endocytosis and recycling of the neurokinin 1 receptor (NK1R) in endothelial cells and spinal neurons. Thus, understanding the inactivation mechanisms of the NK1R is crucial to potential future therapeutic intervention. Therefore, we investigated how different SP concentrations affect NK1R trafficking and determined the role of Rab GTPases in receptor trafficking. NK1R trafficking was markedly influenced by the specific SP concentration. High SP (10 nM) induced translocation of the NK1R and beta-arrestin 1 to perinuclear sorting endosomes containing Rab5a, where NK1R remained for >60 min. Low SP (1 nM) induced translocation of the NK1R to early endosomes located immediately beneath the plasma membrane that also contained Rab5a and beta-arrestin-1, followed by rapid recycling of the NK1R. Overexpression of Rab5a promoted NK1R translocation to perinuclear sorting endosomes, whereas the GTP binding-deficient mutant Rab5aS34N caused retention of the NK1R in superficial early endosomes. NK1R translocated from superficial early endosomes to recycling endosomes containing Rab4a and Rab11a, and Rab11aS25N inhibited NK1R recycling. Rapid NK1R recycling coincided with resensitization of SP-induced Ca2+ mobilization and with the return of surface SP binding sites. Thus, whereas beta-arrestins mediate NK1R endocytosis, Rab5a regulates translocation between early and sorting endosomes, and Rab4a and Rab11a regulate trafficking through recycling endosomes. We have thus identified a new function of Rab5a as a control protein for directing concentration-dependent trafficking of the NK1R into different intracellular compartments and obtained evidence that Rab4a and Rab11a contribute to G protein-coupled receptor (GPCR) recycling from early endosomes. These results increase our knowledge on the mechanisms of GPCR inactivation and may lead to novel

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An ex vivo model for male skin - caffeine conteracts testosterone effects

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Androgenes, especially testosterone and dihydrotesterone influence the skin in various aspects. Some of these androgenic effects result in disadvantages for male skin, e.g. increased transepidermal water loss (TEWL), delayed repair after barrier disturbance and reduced viability of hair at the scalp. Therefore there is a need of substances reversing androgenic effects in skin. To test the antiandrogenic ability of various molecules we invented a male skin organ culture model (MSOCM) based on testosterone-substituted porcine skin. We demonstrate the capability of this model to mimic barrier and proliferation properties of male skin and keratinocytes by investigating TEWL, penetration of nile red, the number of proliferative cells (Ki67positive) and epidermal thickness. Moreover we show that substances with known antiandrogenic properties (zinc, isoflavone) are able to reverse the effects of testosterone in our model, i.e. they reduce the testosterone-induced increase of TEWL and increase the testosterone-induced reduced number of proliferative cells. By using our MSOCM we show that systemically as well as topically applied Caffeine reverses the elevating effect of testosterone on transepidermal water loss and its inhibiting effect on keratinocyte proliferation. We discuss the putative mechanisms of the effects of testosterone and caffeine and the possibilities of application of the latter.

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Interference with the NF-kB pathway in human keratinocytes: different effects on proinflammatory activation and calcium-induced differentiation.

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The transcription factor nuclear factor-kB (NF-kB) has been implicated to be crucial not only for inflammatory processes but also for regulation of proliferation and differentiation. Epidermal homeostasis requires a balance between pro- and antiproliferative events, however, the mechanisms mediating such processes in keratinocytes are incompletely understood. We therefore intended to evaluate the role of the NF-kB pathway for proliferation and calcium-induced differentiation of keratinocytes. Functional blockade of the NF-kB pathway was achieved by retroviral expression of dominant-negative mutants of IKK1 and IKK2. Furthermore, small interfering (si) RNAs knocking-down the NF-kB components p50 and p65 were retrovirally introduced into primary human keratinocytes and HaCaT cells which were then studied with respect to calcium-induced differentiation and TNF-a-mediated proinflammatory activation. Morphology und expression levels of differentiation-associated genes such as involucrin, keratin 5 and 10 were investigated by Western blot and RT-PCR analysis, proinflammatory responses by expression of IL-8 and ICAM-1. Our data suggest that interference at different levels with the NF-kB pathway did not affect calcium-induced keratinocyte differentiation while proinflammatory activation was clearly found to depend on this intracellular signalling pathway.

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CELLS IN THE MURINE DERMIS HAVE IN VITRO CLONOGENIC CAPACITY: CHARACTERIZATION AND ENRICHMENT STRATEGIES

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Evidence exists that the dermis contains multipotent stem cells with the capacity to differentiate into various cell types. However, the phenotype of these cells is still unclear. The aim of our study was to investigate if cell-surface markers present on murine hematopoietic stem and progenitor cells can be used to identify and enrich dermal stem cells. FACS analysis of adult and newborn dermal mouse cells revealed that a small population of CD45+ cells co-expressed Sca-1 and CD117 molecules, suggesting the presence of a reservoir of putative hematopoietic stem and progenitor cells. When testing the in vitro clonogenic capacity of dermal cells in methylcellulose supplemented with cytokines inducing hematopoietic differentiation, we identified different colonies, including adipocytes/sebocytes and colonies with a myeloid cell phenotype. In contrast, peripheral blood failed to generate colonies. Using an adapted, plate-bound technique, we succeeded to highly enrich (purity $\geq$96%; viability $\geq$90%) lineage-depleted dermal cells for Sca-1, CD117, CD34 and CD45. Moreover, all cell populations had the ability to generate colonies. We show for the first time a strategy to phenotype and highly enrich dermal cells with clonogenic capacity. Supported by the Austrian Science Fund P17078-B14.

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Recognition of C. albicans by keratinocytes independent from MyD88 and TLR2/4

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Skin as the outmost barrier takes a prominent position in the protection against microorganisms present in the environment. Here we investigated the role of keratinocytes, which are the main component in the epidermal layer, in the recognition of the pathogenic fungus C. albicans. We have previously shown that the Toll-like receptors (TLRs) 2 and 4 are essential for the recognition of C. albicans by macrophages. As we detected TLR2 and 4 on keratinocytes, we expected that they adopt the same function in keratinocytes. However, recognition of C. albicans, resulting in the activation of the transcription factor NF-κB, was neither impaired in TLR2-deficient nor in TLR4-deficient keratinocytes. MyD88 functions as an adaptor linking TLRs with downstream signalling molecules and is essential for responses against microbial components recognized by TLR2, TLR4, TLR5, TLR7 or TLR9. Activation of NF-κB by C. albicans by MyD88-deficient keratinocytes demonstrates that MyD88-independent pathways are activated in .keratinocytes by C. albicans. These results show that different mechanisms are involved in the recognition of C. albicans at different stages of infection. This may be due to the expression of different pathogen-associated molecular patterns (PAMPs).

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Reduzierte hMSH2 Expression in Melanomzellen nach siRNA Behandlung steigert die Reperaturkapazität der Zellen und die Apoptoserate

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Das menschliche MSH2 ist eine gut charakterisierte Komponente des Fehlpaarungs-DNA Reparatur Systems (MMR). Defekte des MMR sind mit der Entstehung von nicht-polypösen kolorektalen Tumoren assoziiert. In einer Vielzahl dieser Tumore konnten Mutationen in hMSH2 und hMLH1 nachgewiesen werden. Darüber hinaus gibt es jedoch eine Vielzahl von Arbeiten die eine proliferationsassoziierte Überexpression in einer Reihe sporadisch auftretender Tumore beschreiben. Hierzu zählt auch das maligne Melanom. Mit Hilfe der quantitativen "Real time PCR" konnten wir im Vergleich zu nicht malignen Nävi und normalen, kultivierten Melanozyten eine deutlich gesteigerte hMSH2 Expression in Primärtumoren, Metastasen und Melanomzelllinien nachweisen. Die Reduzierung der Expression mit Hilfe von siRNA führt zu einem Anstieg der Apoptose um das 3fache und eine auf 40% reduzierte Überlebensrate verglichen mit nichttransfizierten Zellen. Darüber hinaus wird die Reparaturkapazität der Melanomzellen nach siRNA Behandlung deutlich gesteigert. Transfiziert man einen mit UV-B bestrahlten Reporterplasmid (pCMV-ß-Gal) in eine mit siRNA behandelte Melanomzelle so kommt es zu einer deutlich gesteigerten Reparatur im Vergleich zu einer unbehandelten Zelle. Im Gegensatz dazu zeigen MEF hMSH2 -/- Zellen gegenüber vergleichbaren MEF wt Zellen 24h nach Transfektion eine um 20% verringerte Reparatur. Wir vermuten, dass die gesteigerte Reparatur die Folge eines Zellzyklusarrest ist der durch die Reduzierung der hMSH2 Menge ausgelöst wird. Diese Studie zeigt, dass gegen hMSH2 gerichtete siRNAs effektive Inhibitoren der hMSH2 Expression sind und möglicherweise als Therapeutikum, alleine oder in Verbindung mit anderen Wirkstoffen, eingesetzt werden können.

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Das Vitamin D System in humanen Sebozyten: Relevanz für die Physiologie der Talgdrüse und therapeutische Implikationen

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Neue Forschungsergebnisse belegen eine wichtige Funktion des Vitamin D Stoffwechsels für die Wachstumsregulation in unterschiedlichen Geweben. Unter Verwendung der "real-time" PCR haben wir jetzt in der Sebozytenzellinie SZ95 die Expression von Vitamin D Rezeptor (VDR), Vitamin D-25-Hydroxylase (25-OHase), 25-Hydroxyvitamin D-1α-Hydroxylase (1α-OHase), und 1,25-Dihydroxyvitamin D-24-Hydroxylase (24-OHase) nachgewiesen. Die Inkubation der Sebozytenzellinie SZ95 mit 1,25-Dihydroxyvitamin D führte Dosis-abhängig zu einer Unterdrückung der Zellproliferation. Zusammengefasst belegen unsere Untersuchungen die Expression sowohl des VDR als auch von 1,25-Dihydroxyvitamin D- synthetisierenden und -metabolisierenden Enzymen in Sebozyten. Diese Ergebnisse sprechen für eine physiologische Bedeutung der lokalen 1,25-Dihydroxyvitamin D-Synthese für unterschiedliche Zellfunktionen von Sebozyten, die somit auch aussichtsreiche Zielzellen für eine Behandlung mit Vitamin D-Analoga oder für eine pharmakologische Modulation des 1,25-Dihydroxyvitamin D-Stoffwechsels darstellen.

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Expression of Eph receptors and ephrin ligands in the adult human skin

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Eph receptor tyrosine kinases and their membrane bound receptor-like ligands, the ephrins, represent a bi-directional cell-cell contact signaling system that directs epithelial movements in developmental processes, epithelial wound healing and cancer progression. This family displays a widespread expression in diverse adult human tissues (Hafner et al., Clin Chem 50:490-9, 2004), but the meaning of this system in adult human skin is unknown. Therefore, for the first time, we investigated the expression of selected members of this family in adult human skin.
Immunohistochemical staining was established for EphA2, EphA7, EphB2, EphB4, EphB6, ephrin-A1, ephrin-B1, ephrin-B2 and ephrin-B3. Most of the investigated members were expressed in the adult human skin with apparent restriction to the epithelial compartment (epidermis and follicular adnexes). Interestingly, in contrast, EphA7 is the only member which is predominantly expressed in the wall of blood vessels, the basal membrane zone and musculi arrectores. Some members (e.g., EphB6) show a gradient of expression intensity from the basal cell layer to the corneal layer reflecting different degrees of epidermal differentiation. Additionally, sections of skin ulcers showed enhanced expression of ephrin-A1 in the regenerating epithelial sheet at the edge of the ulcer. Similar to other epithelial cancers, e.g. squamous cell carcinoma of the esophagus, EphA2 is expressed in spinocellular carcinomas of the skin.
Our preliminary data suggest that Eph receptors and ephrin ligands are widely expressed in adult human skin and possibly fulfill functions in epidermal maintenance and organization of epithelial movements. Ongoing studies in our lab try to better define a role of Eph-ephrin-signaling in epithelial wound healing using a HaCaT cell line based scratch wound model.

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Keratinocyte differentiation in the human nail matrix involves apoptosis-like DNA degradation that is independent of activation of capases-3 and 14

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The nail plate is formed by a special differentiation process of keratinocytes of the nail matrix. Similar to the transition of keratinocytes from the stratum granulosum into the stratum corneum, the final stage of the transformation of nail keratinocytes represents a form of programmed cell death. In the present study we investigated whether this process is related to classical apoptosis and/or whether it involves caspase-14, a protease activated in the course of stratum corneum formation. Formalin-fixed paraffin sections of ten adult human nail units were analyzed by immunofluorescence and TUNEL staining. The latter revealed that nuclear DNA of nail matrix keratinocytes was degraded in an apoptosis-like manner in the last living cell layers below the forming nail plate. However, another hallmark of apoptosis, i.e. activation of caspase-3 was undetectable in the entire nail unit. The staining for cytochrome c, whose translocation from the mitochondria to the cytosol is an important step in apoptosis, was uniformely weak or absent not only in TUNEL-positive cells but also in cells at much earlier differentiation stages. This finding is incompatible with a possible function of cytochrome c in the initiation of apoptosis in terminally differentiated nail keratinocytes. Caspase 14 was strongly expressed in all areas of the nail unit where a stratum corneum was formed but was completely absent the nail matrix and the nail bed. Taken together, our data show that the differentiation-associated programmed cell death of keratinocytes in the nail matrix is different from apoptosis and independent of caspase-14.

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Induction of neuropeptide synthesis in skin cells after UV irradiation: a possible mechanism for the development of vascular lesions in rosacea

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Vascular dilatation, which is a characteristic feature of rosacea has still not been adequately explained. The corticotropin-releasing hormone (CRH) family is known to exhibit potent effects on vasculature function, in addition to their critical role in regulation of neuroendocrine and stress response pathways. Neurogenic mediators contribute to inflammation and immunosuppression following UV irradiation of the skin. CRH, the most upstream hypothalamic regulator is also expressed in many peripheral sites, including epidermis and the sebaceous glands, and is upregulated in the skin during inflammation. In this context we investigated the influence of UV irradiation on the synthesis of the major angiogenetic factor VEGF and the neuropeptides CRH and vasoactive intestinal polypeptide (VIP) in human microvascular endothelial cells (HMEC-1), keratinocytes, and SZ95 sebocytes. In addition, we examined the possible paracrine influence of factors derived from epithelial cells by their ability to modify the induction of CRH and VIP. UVA had no effect on VEGF secretion. A physiological UVB dosis induced a dose-dependent decrease of VEGF synthesis in HMEC-1 and SZ95, while in keratinocytes VEGF synthesis was stimulated by UVB irradiation. Culture supernatants modified the UVB effect on VEGF expression of HMEC-1: keratinocyte supernatants inhibited the UVB effect (-25%); SZ95 supernatants annulled the UVB effect; fibroblast supernatants completely protected HMEC-1 against UVB. On the other hand, CRH, CRH receptor I and II, CRH-binding protein and VIP were detected in the cytoplasm of all investigated skin cells by cytochemistry. After UVB irradiation, CRH synthesis significantly increased in keratinocytes, fibroblasts and moderately in SZ95 sebocytes. In endothelial cells factors derived from epithelial cells induced an increased CRH synthesis,only UVB has no effect. Comparable results we found with the neuropeptide VIP, which was also enhanced in HMEC-1 by UVB irradiation. CRH was not secreted in the supernatant, but stored intracellularly. The release pathway of CRH and VIP is unknown but in sebocytes it may be associated with their holocrine secretion. These data suggest that epithelial skin cells respond to environmental stress like UVB by amplified synthesis of CRH, which has direct effects on the vasculature function.

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Caspase-14 derived from stratum corneum of human epidermis is catalytically active

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Caspase-14, a cysteine protease with restricted tissue distribution, is highly expressed in suprabasal epidermal keratinocytes. The proenzyme of caspase-14 is processed to a large and a small subunit during terminal keratinocyte differentiation. To investigate the enzymatic activity of caspase-14, proteins were extracted from stratum corneum of normal human skin. The extracts contained caspase-14 subunits but not procaspase-14 as revealed by Western blotting. By contrast, extracts from parakeratotic epidermis contained both cleavage products and procaspase-14, indicative of incomplete proenzyme activation. A colorimetric assay showed that stratum corneum extracts cleaved synthetic tetrapeptide caspase substrates with a preference for the sequence motif WEHD. The cleavage activity correlated with the concentration of processed caspase-14 and could be removed specifically by immunodepletion of caspase-14. Fractionation of an extract from parakeratotic stratum corneum lead to a partial separation of caspase-14 proenzyme and subunits. The peak caspase activity co-eluted with processed caspase-14 but not with procaspase-14. Our results demonstrate that, in normal epidermis but not in parakeratotic epidermis, the entire pool of endogenous procaspase-14 is converted to proteolytically active caspase-14 subunits.

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Overexpression of S100A15 in psoriatic skin - induction of mRNA expression by Th1 cytokines and Il-1β

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Psoriasis is a chronic inflammatory skin disease affecting appr. 2% of the Caucasian population. Characteristical findings in psoriatic skin are increased proliferation and altered differentiation of keratinocytes, vascular alterations and epidermal infiltration of activated T-lymphocytes and antigen-presenting cells. The etiology of psoriasis is unclear; among others, alterations in cytokine levels have been found. We have recently isolated and characterized a novel member of the S100-protein family, S100A15, which is overexpressed in psoriatic skin and encodes a protein with two Calcium-binding EF-hand motifs. Interestingly, two transcripts of 0.5 kb and 4.4 kb were detected by northern blot analysis. The functional relevance of these mRNA species is still under investigation. The expression and activity of S100-proteins is modulated by the concentration of intracellular calcium. Although the exact biological function of S100-proteins is not fully understood yet, they are assumed to be involved in Calcium-dependent cell cycle progression, differentiation, chemotaxis and innate immunity. S100A15 is likely to participate in the cellular calcium homeostasis and might therefore be an important factor for proliferation and differentiation in psoriasis and other diseases.
In our present study, we examined the influence of relevant Th1 and Th2 related cytokines on S100A15 RNA expression. We performed RT-PCR studies specific for both S100A15 transcripts. Influence of cytokines on gene expression in primary keratinocytes was analysed at different points in time. The S100A15 RNA level was significantly increased by Th1-derived cytokines such as TNF-α and IFN-γ, suggesting a linkage between Th1-lymphocytes and keratinocyte proliferation and differentiation via S100A15-mediated alteration of cellular Calcium-concentration. Moreover, an increase of RNA expression was induced by macrophage-derived IL-1β. Our studies were extended by in-situ hybridisation using probes specific for both mRNA species. We found epidermal localization of both, long and short RNA isoforms in normal and psoriatic skin. Both transcripts were found to be overexpressed in psoriatic skin compared to normal skin. Ongoing analysis regarding the expression of S100A15 in several Th1- and Th2-mediated and tumorous diseases by in-situ hybridisation will further elucidate the biological and pathophysiological role of S100A15.

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Increased cAMP levels interfere with key fibroblast functions

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Through their ability to modulate the expression of extracellular matrix (ECM) components and ECM-degrading enzymes, cytokines, growth factors and various hormones orchestrate the balance between ECM destruction and neosynthesis and therefore play an important role in the control of tissue homeostasis and repair. Cyclic adenosine 3',5'-monophosphate (cAMP), one of the first identified second messengers, transmits signal from a variety of hormones and neuropeptides. Using various molecular approaches to study the consequences of cAMP effects for important fibroblast functions, we have ascertained that cAMP antagonizes prototypic TGF-β-driven plasminogen activator inhibitor type I gene transcription as well as associated protein production in human dermal fibroblasts. TGF-β elicits its effects on target genes through SMAD proteins, which transduce signals from TGF-β receptors into the nucleus where they bind directly to specific promoter sequences. In order to gain insight into the molecular mechanisms underlying the inhibitory activity of cAMP against TGF-β, we demonstrate that increased intracellular cAMP levels prevent TGF-β-induced Smad-specific gene transactivation in fibroblasts, while TGF-β-mediated SMAD nuclear translocation remained unaffected. To assess the physiological relevance of these findings, an in vitro model was used in which TGF-β-treated fibroblasts contracted free-floating collagen lattices. The inductive activity of TGF-β on the capacity of fibroblasts to contract mechanically unloaded collagen lattices was significantly suppressed by increased intracellular cAMP levels. Furthermore, synthetic cAMP treated fibroblasts exhibited significantly lower ability to close mechanically induced cell layer wounds than their untreated counterparts. Together these results identify cAMP as a potent regulator of TGF-β-mediated key fibroblast functions important for excessive scar contraction and development of fibrotic disorders.

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The distal C-terminus of BP180/collagen XVII is important for ligand binding and integration into the cell membrane.

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Collagen XVII, a transmembrane component of the hemidesmosomes, mediates adhesion of the epidermis to the underlying basement membrane by binding to laminin 5. Genetic defects of collagen XVII are associated with epidermal detachment in junctional epidermolysis bullosa (JEB). Our recent studies revealed a spontaneous deletion of the distal C-terminus of collagen XVII in a JEB patient. This mutation was a homozygous duplication of four nucleotides in exon 54 of the COL17A1 gene, which resulted in an adjacent nonsense sequence of 18 amino acids with a premature termination and the elimination of 43 most C-terminal amino acids of the collagen XVII ectodomain. However, the mutation led to expression of a truncated molecule, albeit at a somewhat lower level of about 60% of the wild-type controls. For further experiments an eukaryotic expression vector for the C-terminal deleted collagen XVII was constructed and used for transfection studies and production of recombinant protein. Transfection of COS-7 cells with mutant collagen XVII results in its intracellular accumulation, shown by immunoblot analysis of the cell lysates. Analysis of the thermal stability by limited trypsin digestion showed that the helix-to-coil transition temperature was significantly lower for the truncated collagen XVII than for wild type controls, indicating abnormal folding of the mutant molecule. Immunhistological studies on JEB keratinocytes by double immunofluorescence labeling showed an aberrant scattered deposition of laminin 5 and collagen XVII away from the cell bodies. In addition, JEB keratinocytes and mutant collagen XVII transfected COS-7 cells showed an accumulation of their collagen XVII precursor products within the entire intracellular space. In contrast, wild-type cells showed a restricted perinuclear localization of collagen XVII precursor in lower quantities. Analysis of cell motility by time-lapse videomicroscopy revealed that JEB cells were more motile than normal keratinocytes, but in an undirected manner. Comparable motility characteristics have been observed before with collagen XVII deficient keratinocytes. These findings demonstrate that the deletion of the laminin 5-binding domain of collagen XVII alters physiologic ligand binding and cell motility and causes abnormal folding and accumulation of the precursor within the cell. These results give the first indication of an involvement of the distal ectodomain of collagen XVII for protein targeting.

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cIAP2 is not the critical mediator for resistance to tumor necrosis factor (TNF)α-induced apoptosis in human keratinocytes

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The regulation of apoptotic pathways is of major importance for tissue homeostasis of human skin. Death ligands like TNFα and TNF-related apoptosis inducing ligand (TRAIL) activate the apoptotic machinery, but also initiate inflammatory responses. However, whereas TRAIL and TNFα induce NF-κB, only TRAIL induces apoptosis in human keratinocytes. In contrast, when NF-κB activation was inhibited via ectopic expression of a dominant-negative mutant of IKK2 (HaCaT-IKK2-KD), keratinocytes were dramatically sensitized to TNFα-induced apoptosis. This sensitization was neither explained by differences in cell surface expression of TNF receptors nor by basal expression of initiator caspases 8 or 10 but occurred at the TNF receptor complex. Accordingly, sequential retroviral infection of the caspase 8 inhibitor cFLIPL fully inhibited TNFα-induced cell death in HaCaT-IKK2-KD. To further pinpoint potential molecules responsible for sensitization to TNFα in HaCaT-IKK2-KD, we investigated a panel of NF-κB-regulated effector molecules. Interestingly, the inhibitor of apoptosis (IAP) family member cIAP2, but not cIAP1, XIAP or TRAF2 was downregulated in HaCaT-IKK2-KD. Knock-down experiments targeting c-IAP2 by retroviral siRNA transduction of HaCaT revealed that despite efficient knock-down of cIAP2 mRNA, resistance to TNFα-induced apoptosis was maintained. These data indicate that cIAP2 alone is not sufficient to sensitize keratinocytes for TNFα-mediated apoptosis.
In conclusion, we demonstrate that inhibition of NF-κB dramatically sensitizes human keratinocytes to TNFα-induced apoptosis at the level of caspase 8 activation. Moreover, our data provide evidence that the NF-κB-dependent modulation of cIAP2 is not critically required for this process. This suggests that other known or unknown NF-κB target molecules are likely to be more important for the resistance to TNFα-mediated apoptosis in keratinocytes. These molecules may thus deviate the biological outcome of TNF receptor stimulation in human skin.

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Interaction of androgens and PPAR ligands in human sebocytes

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Androgens apparently play a crucial role in increasing synthesis of sebaceous lipids and in triggering the initiation of sebaceous gland-associated diseases in humans, such as acne vulgaris. On the other hand, androgens in vitro stimulate sebocyte proliferation, while lipid synthesis remains unchanged. This contradiction leads to the assumption that co-factors may be required for the induction of the in vivo detected androgen effect. Current research has indicated that peroxisome proliferator-activated receptors (PPAR) and their ligands, known to be involved in adipocyte differentiation, could be the primary candidates. Using the immortalized human sebaceous gland cell line SZ95 we examined the influence of testosterone (2x10-8 M) and linoleic acid (10-4 M), a PPAR ligand, as single agents and in combination on lipid content and 5α-dihydrotestosterone (DHT) synthesis by means of nile red microassay and enzyme-linked immunosorbent assay respectively. We found that testosterone alone, as expected, has no effect on sebocyte lipid content, while linoleic acid significantly increased neutral (sebocyte) lipids (+523%, $p<0.005$ to control) and polar (membrane) lipids (+50%, $p<0.005$ to control). The combination of testosterone and linoleic acid exhibited a synergistic effect by increasing neutral lipids (+599%, $p<0.005$ to control and $p<0.01$ to linoleic acid) and polar lipids (+59%, $p<0.005$ to control, ns to linoleic acid). Furthermore, DHT was increased in culture supernatants under treatment with testosterone and linoleic acid in comparison with the treatment with testosterone alone. To further corroborate our findings, we examined the influence of the 5α-reductase inhibitor LY 191704 in similar experiments. Sebocyte lipid production and DHT synthesis were not affected after incubating the cells with LY 191704 (10-8 M) and testosterone or linoleic acid, but neutral lipids were decreased (-30%, $p<0.005$ to control) and DHT synthesis was inhibited (-29%, $p<0.01$ to control) after treating the cells with all three compounds, suggesting that LY 191704 could only unfold its action in the presence of both testosterone and linoleic acid. These data implicate the synergistic action of androgens and PPAR ligands on lipid stimulation and DHT synthesis in human sebocytes, elucidate the mode of androgen activity in vivo and can be used as an in vitro model for further studies with androgen antagonists and acne treatment.

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The role of E-cadherin mediated cell-cell adhesion in epidermal barrier formation

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The cadherin family of Ca-dependent adhesion molecules are important mediators of tissue morphogenesis. Next to their role in cell-cell adhesion, cadherins have been implicated in many other processes such as cell differentiation, proliferation, cytoskeletal regulation and polarity. To analyse the function of E-cadherin in skin, we have specifically inactivated E-cadherin in mouse epidermis using the Cre-loxP system. We chose the epidermis because it is a self-renewable tissue, and E-cadherin is the only classical cadherin expressed in all layers of the epidermis. E-cadherin mutant mice revealed a shiny and parchment-like appearance with increasing scaling over time, indicating a disturbed epidermal barrier. Surprisingly, using toluidine blue staining and lucifer yellow penetration assays, no obvious difference could be observed in the outside-in epidermal barrier between control and E-cadherin mutant mice. In contrast we did find a defect in the inside-out barrier of the E-cadherin mutant mice using dermally injected biotin as a tracer. These results showed that E-cadherin is essential for epidermal barrier function. To understand the mechanism by which E-cadherin contributes to barrier formation, we isolated primary keratinocytes from control and E-cadherin mutant mice. Initial analysis of these keratinocytes revealed no gross alterations in growth properties. In addition, we did not find any significant differences in other adherens junction protein expression using western blot analysis. Immunofluorescence analysis of Ca2+-switch assays suggests that the E-cadherin deficient keratinocytes are delayed in their formation of cell junctions, using b-catenin as an adherens junction marker and ZO-1 as a tight junctional marker. These experiments also showed that P-cadherin, normally confined to the basal layer of epidermis, was still expressed in the differentiating keratinocytes, suggesting partial compensation for the loss of E-cadherin. To test this hypothesis we are presently setting up RNAi experiments in primary keratinocytes. In addition, we will examine the migratory properties of the E-cadherin deficient keratinocytes and use biochemical methods to analyse cell junctional complex composition.

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INTERACTION OF SIGNALING PATHWAYS DURING CAPACITATION OF HUMAN SPERMATOZOA

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Objective: Capacitation of spermatozoa is a prerequisite for successful fertilization, although the exact molecular mechanism is not fully clarified. The calpain(CLP)-calmodulin(CM)-system is assumed to be essential for membrane fusion during this process. Activation of caspases (CP) is a main feature of apoptotic cells. As CP1 triggers CLP, both signaling pathways may be cross-linked. The objective of our study was to examine interactions of apoptosis signaling pathways and the calpain-calmodulin-system during capacitation.

Design: A prospective study

Material and Methods: Semen samples of donors (n=14) were subjected to a density gradient centrifugation to retain motile and mature spermatozoa. Four aliquots were incubated in BWW at 37°C 5% CO2 for 3h with: no additive (control); 3% BSA (capacitation); 10μM calpain inhibitor III (CLP-inh) or 20μM calmodulin inhibitor (Ophiobolin A, CM-inh). Linear Motility (Mota) and hyperactivated spermatozoa (HAS, seen in capacitated sperm only) were assessed by CASA. In addition capacitation was monitored by CTC-assay (CTC-A=non-capacitated sperm) and tyrosine phosphorylation (Western blot). FACS analyses were performed to evaluate CP1,-9 and -3 activation (fluorescence labeled inhibitors of caspases) and the integrity of transmembrane mitochondrial potential (iTMP, lipophilic cationic dye).

Results:
Parameter: control - capacit. - CLP-inh - CM-inh
CTC-A: 48.5±20.9; 28.3±9.7; 38.9±8.6*; 65.5±19.0#
HAS: 2.3±1.9; 14.0±8.0; 6.6±6.1*#; 0.9±1.8*#
Mota: 16.1±10.6; 33.3±12.2; 34.3±14.6*; 4.0±6.8*#
CP1: 21.7±15.9; 11.8±8.2; 10.7±8.0; 74.7±27.2*#
CP9: 19.5±16.9; 9.7±6.3; 10.8±6.6*; 77.7±25.7*#
CP3: 15.6±3.0; 10.8±8.3; 9.3±13.0; 78.6±25.2*#
iTMP: 70.9±16.0; 88.4±7.7; 88.5±8.8*; 19.8±20.5*#
% spermatozoa, mean±SD, *p<0.05 (CLP-,CM-inh vs. control) #p<0.05 (CLP-,CM-inh vs. capacitation).

Conclusion: Capacitated sperm are characterized by lowest caspase activation levels and preserved mitochondrial potential integrity. Inhibition of calpain during capacitation reduced significantly capacitation related parameters, but did not lead to apoptosis. Inhibition of calmodulin resulted in both: blocking of physiological changes seen during capacitation and apoptosis. Interaction of both signaling systems seems to enable the capacitation process by prevention of apoptosis.

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Microbial proteases with lysine specificity activate protease-activated receptor 1 (PAR1) pathway in keratinocytes

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Previous studies revealed that the lysine-specific protease IV from Pseudomonas aeruginosa stimulates the production of proinflammatory mediators IL1 beta, IL-8 and TNF alpha as well as the antimicrobial peptides HBD-2 and RNase-7 in HaCaT keratinocytes. This stimulation was dependent on Ca2+-influx and was shown to involve the protease-activated receptor 1 (PAR1) pathway.

Now we investigated a variety of bacterial and fungal proteases regarding their ability to stimulate Ca2+-influx in keratinocytes: endoproteinase Lys-C from Lysobacter enzymogenes, endoproteinase Glu-C from Staphylococcus aureus, proteinase K from Tritirachium album, and subtilisin A from Bacillus subtilis. With exception of endoproteinase Glu-C these proteases induced Ca2+-influx in HaCaT keratinocytes. The keratinocyte Ca2+-influx-inducing activity correlated well with the catalysis of the chromogenic substrate tosyl-glycyl-prolyl-lysine-4-nitroanilide of the respective proteases, indicating that proteases were also competent to cleave lysyl-carboxyl-bonds. The deactivation of protease-mediated Ca2+-influx of subtilisin A and proteinase K by prestimulation with thrombin points towards an involvement of protease-activated-receptor 1 in keratinocyte activation by these proteases.

These results show, that proteases from bacterial sources with specificity for lysyl-carboxyl-bonds may play a role in the epidermal recognition of microorganisms and the regulation of the adaptive innate immune system. Thus, lysine-specific proteases from microorganisms may be regarded as a family of pathogen associated molecules (PAMs) which signal the presence of microorganisms on epithelia and induce various innate immune responses in order to facilitate their elimination.

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The actin-ECM (extracellular matrix) linkage defects caused by mutations in the Kindlin1 gene are mediated by Actinin alpha

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The kindler syndrome characterized by congenital blistering, photosensitivity, and skin fragility is caused by mutations in the kindlin1 gene. The majority of the disease associated mutations lead to truncated protein products, which are lacking the FERM and PH (Pleckstrin) domains. In cell-cell and cell-ECM type adherent junctions several proteins such as vinculin, talin, actinin alpha are involved in the anchorage of F-actin to the membrane. The involvement of kindlin1 in the anchorage of F-actin was not clear, however, the observed ECM linkage defects and colocalization of kindlin1 with vinculin as well as the association of kindlin1 with filamentous actin supported such a view. In a global yeast two-hybrid screen with a FERM/PH containing fragment of kindlin1 gene as the bait, we could identify actinin alpha as an interacting protein. Interestingly the interacting fragment of actinin alpha is reduced to the spectrin domain containing part of the protein thereby indicating a link between disease causing mutations and the molecular interaction of actinin alpha spectrin domain with the FERM/PH domain of kindlin1. Since the actinin alpha interacts specifically with vinculin, filamentous actin and kindlin1 which are all in the immediate vicinity of actin-membrane anchorage sites, we propose that the skin fragility disorder kindler syndrome is an actin-ECM linkage defect. Confirmation of the yeast two-hybrid positive interaction was done by immuneprecipitation of in vitro transcriped/translated kindlin1 protein and actinin alpha protein. Further verification of the protein-protein interaction was performed by a novel computational protein interaction prediction methode which is based on conserved sequence alignments

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α-Melanocyte-stimulating hormone does not interfere with transforming growth factor-β1-induced signal transduction but modulates mRNA expression of several newly identified target genes

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α-Melanocyte-stimulating hormone (α-MSH) has pleiotropic effects on a variety of cutaneous cell types. These effects are mediated by melanocortin receptors which belong to the superfamily of G-protein-coupled receptors and couple ligand binding to activation of adenylate cyclase thereby increasing intracellular cAMP levels. We recently reported that α-MSH suppresses the profibrotic activity of transforming growth factor-β1 (TGF-β1) on collagen synthesis in human dermal fibroblasts in vitro as well as in a mouse model of cutaneous fibrosis. Since cAMP can affect a number of signal transduction pathways, we investigated whether α-MSH interferes with TGF-β1-induced activation of the mitogen-activated protein kinase (MAPK) cascade and the smad pathway in human dermal fibroblasts. In contrast to epithelial cells, TGF-β1 failed to induce MAPK1/2 in serum-deprived cells and α-MSH per se did not activate these kinases either. TGF-β1-induced activation of smad2/3 - as judged by nuclear translocation of smad2/3 and serine phosphorylation of smad2 remained unchanged by alpha-MSH. Furthermore, alpha-MSH did not alter the relative mRNA levels of smad7 induced by TGF-β1. Since these studies left open the molecular mechanism by which α-MSH affects collagen expression by TGF-β1, we performed high-density oligonucleotide arrays using Affymetrix HuGeneFL gene chips covering 5600 cloned genes. Real-time PCR analysis was used to validate significantly regulated genes as well as to monitor expression of selected genes strongly implicated in fibrogenesis and extracellular matrix composition. Accordingly, α-MSH modulated the expression of several genes induced by TGF-β1, e. g. connective tissue growth factor, matrix metalloproteinase 1, elastin, tissue factor pathway inhibitor 2, fibroblast growth factor 1, early growth response 1, as well as members of the IL-6 family of proinflammatory cytokines. Our findings may provide clues to the molecular mechanism by which α-MSH modulates the effect of TGF-β1 in fibroblasts and point towards novel biological activities of this neuropeptide.

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Assessment of acute effects of sidestream cigarette smoke on HaCaT-keratinocytes in vitro

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Sidestream cigarette smoke (SS) can be regarded like other environmental factors as an external toxic factor for the skin. In the present investigation the acute effects of SS are assessed in vitro by means of the neutral red dye release assay (NRRA).
Confluent HaCa-T keratinocytes were incubated 3h with a neutral red solution before a 15 min exposure to SS under a supernatant of PBS. SS was collected from three University of Kentucky research cigarettes (2R4F) immediately prior to exposure. After exposure the cells were destained and the optical density (OD) of the resulting solution was measured at 540 nm. To assess whether the amount of supernatant affects the toxicity of SS, cells were exposed to SS under different amounts of PBS ranging from 0 to 3000µl. To investigate if SDS affects the sensitivity of the cells to SS, cells were exposed to SDS before and after SS exposure. In a further experiment the cells were incubated with a solution containing 50µg/ml vitamin C before smoke exposure. Finally cells were incubated with different SDS concentrations ranging from 0,2µmol to 1,0 µmol prior to smoke exposure to evaluate dose dependent effects of SDS-related changes of the sensitivity of the cells to SS.
The results obtained demonstrate that the amount of supernatant during exposure affects the toxic effects of SS which were strongest with a supernatant of 1000µl PBS. In consequence all following experiments were performed with a supernatant of 1000µl. Incubation of the cells with SDS before smoke exposure decreased OD up to 5,5fold in comparison to smoke alone. Incubation of the cells with SDS after smoke exposure decreased OD up to 1,87fold in comparison to smoke alone. The OD assessed after incubation with vitamin C was 1,30fold higher than after SS alone. SDS-induced increases of the sensitivity to SS increased up to 0,8 µmol SDS.
In conclusion the results obtained indicate that a strong acute toxicity of SS can be assessed by the NRRA. Since the NRRA assesses membrane alteration it can be concluded that the effects of SS are at least in part due to effects on membrane structures. The fact that vitamin C decreases the effects of SS indicates that oxidative mechanisms appear to be involved. Further research is needed to elucidate the mechanisms underlying these findings and to evaluate the clinical impact of these findings.

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The molecular mechanisms of early hair follicle development : The role of the EDA- A1/EDAR/NF-κB signaling pathway

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The transcription factor family NF-kB/Rel plays an important role in many cellular events such as cell cycle and cell death, but also in processes of the immune response. We have generated mice expressing a transdominant negative mutant of the NF-kB inhibitor IkBa, to block NF-kB activation. IkBaDN cDNA was integrated in frame into the ubiquitously expressed b-catenin gene. Knock-in IkBaDN mice revealed defects in the early development of most hair follicle types, exocrine glands and teeth identical to those of mice with mutations in the tabby (ta; eda) and downless (dl; edar) locus, and present, thus, an analogous phenotype to humans suffering from a rare hereditary ailment called HED (Hypohydrotic Ectodermal Dysplasia). EDA-A1 and EDAR are related to the TNF multigene family of ligands and receptors, respectively, which are known to activate NF-kB. To confirm NF-kB activity in these structures we analyzed mice expressing an NF-kB-responsive b-galactosidase construct ((Igk)3conalacZ) which showed strong NF-kB activity in these structures as early as E14 for pelage hair, which coincides exactly with the first localized EDAR expression in placodes. Thus, the EDA-A1/EDAR/NF-kB signaling pathway starts at E14.5. With the exception of b-catenin, EDA-A1 and EDAR, expression of important regulators of hair follicle development such as Wnt10b, Shh, BMP#s and Lef-1 are absent in guard hairs of IkBaDN mice, but not in the secondary awl hairs. We demonstrate that NF-kB is needed for ectodermal placode formation of guard hairs at the initial stage, and that it is directly activated downstream by EDA-A1, interacting with its receptor EDAR. The first mesodermal or ectodermal signal Wnt/b-catenin leads to localization of EDAR to the future placode site, and subsequent binding of EDA-A1 leads to activation of NF-kB. Direct or indirect targets of NF-kB activity such as Wnt10b, 10a,or Shh lead to the proliferation of the hair placode and later to morphogenesis of the hair follicle. Furthermore, we can show that NF-kB is important for maintaining of the epidermal structure. It is not needed for secondary awl hair development, but for zigzag morphogenesis.

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