O1

Basic Fibroblast Growth Factor (bFGF) is a Critical Microenvironmental Factor in Human Skin for the Induction of Melanoma by UVB

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UV light is an epidemiological risk factor for melanoma, but its specific contribution to melanoma induction is not known. The critical first step of melanoma development, i.e. the uncontrolled proliferation of melanocytes, may be induced by a combination of UV damage and an imbalance of growth factor production by cells in the immediate environment of the melanocyte. Among several tested cutaneous growth factors (HGF, IGF-1, PDGF-A, bFGF), overexpression of bFGF via adenoviral gene transfer in human skin xenografted to SCID mice led to the most striking effects on the pigment cell system. Black pigmented macules developed within 3 weeks of bFGF treatment. Immunofluorescence for TRP-1, HMB-45, and Ki-67 demonstrated pathological hyperpigmentation, proliferation and hyperplasia of activated melanocytes, but no malignant transformation. On the other hand, when bFGF was combined with UVB, pigmented lesions with hyperplastic melanocytic cells were detected including a lesion with high-grade atypia resembling acrolentiginous malignant melanoma *in situ*. Donor-matched control grafts had no melanocyte changes. This is the first observation that an imbalance of physiological growth factor production in the skin can cause melanoma in combination with UVB. Because bFGF was found overexpressed in the dermal fibroblasts, global gene expression analyses of bFGF-transduced fibroblasts embedded in collagen were performed to identify candidate paracrine factors activated by bFGF. The melanocyte mitogen endothelin-3 was upregulated suggesting that additional physiological factors may also contribute to melanoma development.

O2

Recessive mutation in desmoplakin disrupts desmoplakin/intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma.

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Desmosomes are major cell adhesion junctions, particularly prominent in the epidermis and cardiac tissue, and are important for the rigidity and strength of the cells. The desmosome consists of several proteins, of which desmoplakin is the most abundant. Here, we describe the first recessive human mutation, 7901delG, in the desmoplakin gene which causes a generalised striate keratoderma particularly affecting the palmoplantar epidermis, woolly hair and a dilated left ventricular cardiomyopathy. Many of the patients with this syndromic disorder suffer heart failure in their teenage years, resulting in early morbidity. All tested affected members of three families from Ecuador were homozygous for this mutation which produces a premature stop codon leading to a truncated desmoplakin protein missing the C domain of the tail region. Histology of the skin revealed large intercellular spaces and clustering of desmosomes at the infrequent sites of keratinocyte adhesion. Immunohistochemisty of skin from the patients showed a perinuclear localisation of keratin in suprabasal keratinocytes suggesting a collapsed intermediate filament network.

This study demonstrates the importance of desmoplakin in the attachment of intermediate filaments (IFs) to the desmosome.

O3

A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by $CD4^+$ cytolytic T lymphocytes.

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Antigens encoded by *MAGE-A3* and recognized by T cells are interesting targets for tumor immunotherapy because they are strictly tumor-specific and shared by many tumors of various histological types. A number of MAGE-A3 antigenic peptides presented by HLA class I molecules have been used in clinical trials and regressions of melanoma metastasis have been observed. We report here the identification of a MAGE-A3 epitope presented to $CD4^+$ T lymphocytes by HLA-DP4 molecules, which are expressed in approximately 72% of Caucasians. To identify this epitope, monocyte-derived dendritic cells were loaded with a recombinant MAGE-A3 protein and used to stimulate autologous $CD4^+$ T cells, obtained from blood donors without cancer. We isolated a $CD4^+$ T cell clone that recognized peptide TQHFVQENYLEY. Interestingly, the $CD4^+$ T cells lysed HLA-DP4 tumor cells expressing *MAGE-A3*, indicating that this epitope, in contrast to other class-II MAGE-A3 epitopes, is presented at the surface of tumor cells. Moreover, in a vaccination trial where patients with metastatic melanoma were immunized with peptide-pulsed dendritic cells the majority of patients developed TH1 immunity to this epitope as revealed by ELISPOT analysis.

O4

Functional Role of the Sodium-Hydrogen Antiporter, NHE1: Studies of NHE1 Null-Allele Mouse Epidermis.

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The external layers of the epidermis, the stratum corneum (SC), shows an outward-directed, increasingly acidic pH. Neither the origin, or the function of this gradient in normal cutaneous function and/or disease are understood. We reported previously that: 1) permeability barrier recovery in tape-stripped hairless mouse proceeds normally at an acidic pH, but is delayed at a neutral pH (i.e. pH 7), due to disturbed post-secretory, extracellular lipid processing (ADR, 290:215, 1998); 2) sodium-hydrogen exchanger protein (NHE1) is present in cultured human keratinocytes (CHK) and in the outer nucleated layers of human epidermis; and 3) CHK recovery from an acid load (i.e. H^+ export) is impeded by low-dose (10^{-6} M) amiloride, suggesting that NHE1 functions to regulate both intra- and extracellular pH (pH_i, pH_e). To determine more precisely the role of the NHE1 antiporter in pH regulation and subsequent SC acidification, we used both pharmacologic and molecular biology approaches. First, we used the more specific NHE1 inhibitor, HOE694, which blocked recovery of CHK pH_i similarly to amiloride. Topical administration of HOE694 to hairless mice skin immediately following tape-stripping also produced a pronounced delay in permeability recovery (by transepidermal water loss), which pH 5.5 buffer reversed, but not pH 7.4.

Finally, adult NHE1 null (-/-) mice also showed a delay in barrier recovery following tapestripping, alterations in lamellar body secretion and post-secretory processing of extracellular lipids to mature extracellular lamellar membrane structures, and increased SC thickness.

In summary, pharmacologic inhibition of NHE1 revealed the importance of the specific H^+/Na^+ antiporter, NHE1, for pH_i, and barrier recovery, while alterations of SC structure and function in NHE1 null mice reveal its necessity for normal barrier homoeostasis.

O5

Germline and somatic CDKN2A mutations in multiple primary melanoma patients.

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Patients with multiple primary melanomas (MPM), who develop several concomitant or successive primary melanomas, often have a family history of melanoma. In recent studies, germline CDKN2A mutations in MPMseries have been correlated to the familial occurrence rather than to the melanoma multiplicity itself. In contrast, the genetics of patients with MPM in the absence of a family history of thedisease has not yet been investigated in detail. In order to clarify the presence of genetic predisposition to the development of multiple melanomas, we analyzed the CDKN2A gene for germline and somatic mutations in four patients with two or more primary melanomas and no evidence of a family history of the disease. Polymerase chain reaction and direct DNA sequencing were used to screen the CDKN2A gene for germline mutations. Loss of heterozygosity (LOH) and microsatellite instability (MSI) studies at 9p21(D9S974, D9S126, D9S171) were performed in tumor specimens of each MPMpatient in laser microdissected sections by amplification of (CA)n repeat units, followed by electrophoresis of the PCR product and hybridization with ^{33}P-end-labeled oligonucleotides. Mutational analysis of genomic DNA showed a germline missense mutation in exon 2 (Gly101Trp) of the CDKN2A gene in one MPM patient who had seven independent primary melanomas resected.LOH at D9S974 has been identified in 2/7 melanoma tissues of the same patient. In addition, one of the two daughters of the proband carried theCDKN2A mutation. Finally, LOH at D9S171 was detected in 1/4 melanomas of a MPM patient who did not show any germline CDKN2A mutation. Based on our results, the presence of germline mutations of the CDKN2A gene in patients with MPM but no family history is highly suggestive of a genetic predisposition to the development of multiple melanomas. In addition, our data support the pathogenetic role of bi-allelic inactivation of the CDKN2A gene in melanoma development.

O6

ADAMs may be involved in the physiological shedding process of human collagen XVII.

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Collagen XVII is a type II transmembrane protein which occurs as a full length 180 kDa protein and as a soluble 120 kDa form. Time chase experiments revealed that the soluble ectodomain was first detectable in the medium after 9 min and was stable for more than 48 hours. New antibodies raised against recombinant fusion proteins of the NC16A domain and the last 50 amino acids of the C-terminus of collagen XVII demonstrated that the authentic shedding product extends from the NC16A domain to the C-terminus of the collagen XVII molecule. The shedding was stimulated by PMA and IL-1<FIELD symbol><98 \f "Symbol" \s 11≠β>, stimulators of matrix metalloproteinases and ADAMs. To characterize the proteases involved in the shedding, normal human keratinocytes were cultured in the presence of protease inhibitors. The shedding was completely inhibited by phenanthroline, staurosporine, MMP- and sheddase-specific hydroxamates (BB 3103, BB 3241 and TAPI), but not by the selective gelatinase inhibitor CTTHWGFTLC and serine protease inhibitors. Further experiments revealed that recombinant MMP-2, MMP-9 and MT1-MMP cleaved immunoprecipitated collagen XVII only to a specific product of about 140 kDa, which showed positive immunoreactivity with an endodomain specific antibody and that a MMP2-deficient human gastric carcinoma with very low MT1-MMP expression, showed normal ectodomain shedding. Taken together all these results, it could be assumed that MMP-2, MMP-9 and MT1-MMP may not have physiological importance in collagen XVII shedding. Since we are able to detect the expression of the disintegrin/metalloproteases ADAM-10 and ADAM-17 (TACE) in human keratinocytes on mRNA and protein level, it could be speculated that these proteases are involved in the collagen XVII processing from keratinocyte surface. In addition, it could not ruled out that a multiproteinase cascade, consisting of furin-like proteases, metalloproteinases and ADAMs/sheddases, is involved.

O7

α-Melanocyte Stimulating Hormone (α-MSH) as a Survival Factor in Hypoxia.

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By virtue of location and ability to synthesize various anti-inflammatory as well as vasoactive mediators, the vascular endothelium plays a key role in inflammation and in the maintenance of vascular homeostasis. The neuropeptide α-MSH is a potent inhibitor of inflammation, and endothelial cells (EC) are known to be a target for as well as a source of α-MSH. Hypoxic stress activates EC to release growth factors and pro-inflammatory mediators. However, little is known about the characteristics of EC death in response to hypoxia.

The aim of the present study was to test the ability of αMSH to influence the survival of human dermal microvascular endothelial cells (HDMEC) under hypoxic conditions. HDMEC were cultivated under hypoxic conditions (0.5% O_2) for 24h +/- different concentrations of α-MSH (10^{-8}-10^{-12} M). Cell viability was tested using a MTT based assay and the α-MSH precursor proopiomelanocortin (POMC) mRNA expression was determined by quantitative RT-PCR. We observed that hypoxia significantly reduces cell viability in HDMEC. Treatment with 10^{-8} M α-MSH prevents hypoxia-induced cell death. The expression of HDMEC POMC mRNA is markedly increased under hypoxic conditions. These data provide first evidence that α-MSH, in addition to its anti-inflammatory capacity, may serve as a survival factor during hypoxic conditions and suggest that it could be used therapeutically in various diseases like vasculitis, chronic wound healing and tissue ischemia.

O8

Apo2L/TRAIL-dependent Recruitment of Endogenous FADD and Caspase-8 to Death Receptors 4 and 5.

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Apo2L (also called TRAIL) is an apoptosis-inducing member of the tumor necrosis factor (TNF) family. Numerous tumors (including squamous cell carcinoma) are sensitive to apoptosis-induction by Apo2L/TRAIL, but most normal cells (including keratinocytes) are relatively insensitive. To begin to investigate the basis for differential sensitivity to Apo2L/TRAIL we wished to determine its apoptosis signaling mechnism. The ligand triggers apoptosis through two distinct death receptors, DR4 and DR5. Receptor overexpression studies have yielded conflicting results on the involvement of certain factors in Apo2L/TRAIL function. Here we show in non-transfected cells that Apo2L/TRAIL induces homomeric and heteromeric complexes of DR4 and DR5, and stimulates recruitment of the adaptor molecule FADD and the protease caspase-8, as well as caspase-8 activation. In contrast, the adaptor molecules TRADD and RIP, which bind to TNF receptor 1, did not bind DR4 and DR5. Thus, Apo2L/TRAIL initates apoptosis through a mechanism similar to that used by the death ligand FasL, and FADD may be a universal adaptor for death receptors.

O9

The Influence of UVA and UVB on the Tyrosine Kinase Profile of Normal Human Keratinocytes.

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Protein tyrosine kinases (PTKs) play a significant role in signalling pathways that regulate cell proliferation, differentiation, transformation and immune responses. After exposure to stress PTKs have been described to be involved in the induction of growth arrest and apoptosis. Exposure of human skin to UV induces major changes in the genetic program of the exposed cells leading to immediate and long term skin changes. Although it can be assumed that UV-induced modifications of signal transduction involving PTKs regulate these processes, details as to the specific changes in PTK expression after UV exposure are unknown.

To investigate PTK expression in normal human keratinocytes (HNK) we employed a reversed transcriptase-PCR approach using degenerate primers derived from the conserved catalytic domain of PTKs. PCR products were cloned and PTKs from randomly picked colonies (up to n = 90 per screen) were identified by sequence analysis. PTK profiles of sham-irradiated, UVA (filtered metal halide lamp, 60 J/cm^2), and UVB (filtered metal halide lamp, 256 mJ/cm^2) treated HNK were analyzed 7 h after exposure. Results were analyzed by ÷^{2} test for statistical significance.

We identified 13 PTKs including 3 receptor kinases (*axl, cak, fgfr*) and 10 nonreceptor kinases (*abl1/2, ick, map4k2, fyn, yes, src, cnk, ptk6, mstr1, jak*). The PTK profile of HNK was characterized by a predominance of *abl1/2* (46% of PTKs). Differential screening revealed a further induction of *abl1/2* expression by UVA (84 %). UVB had no influence on *abl1/2* but predominantly induced the expression of the receptor kinases of the *axl*-family. The differences reached statistical significance at $p<0.0001$.

These results for the first time provide information on the PTK expression profile of HNK and its modification by UV. The observed UV effects are wavelength dependent and affect PTKs which are involved in the regulation of gene transcription, cell death, and proliferation. We conclude that regulation of PTK expression is part of genetic program that mediates late effects of UVA and UVB through specific alterations in phosphorylation dependent signalling cascades.

O10

Loss of expression of *p16^{INK4a}* and *p14^{ARF}* genes via promoter hypermethylation in human nonmelanoma skin cancers.

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The *INK-ARF* locus localized on human chromosome9p21 encodes two alternative reading frame proteins (*p16^{INK4a}* and *p14^{ARF}*) known to function as tumor suppressors via the retinoblastoma or p53pathway. Inactivation of *p16^{INK4a}* can lead to dysregulation of these two pathways. Although mutations inthe p53 gene are uncommon in human melanoma, loss of the tumor suppressor activity of *p16^{INK4a}* in familial and sporadic melanoma is well documented. In addition, inactivation of *p16^{INK4a}* involving mutations, deletions or promoter hypermethylation has been found in a variety of human tumors. However, it is not clear whether genetic alterations in *p16^{INK4a}* and *p14^{ARF}* play a role in the development of human nonmelanoma skin cancer (NMSC). We, therefore, analyzed 40NMSC (21 primary squamous cell carcinomas, 17 basal cell carcinomas and 2 actinic keratoses) for mutations in *p16^{INK4a}* and *p14^{ARF}* genes using PCR and SSCP techniques. Nomutations in either the *p16^{INK4a}* or the *p14^{ARF}* gene were found in these tumors. However, immunohistochemical analysis revealed loss of expression of p16 and p14 proteins in 97% of NMSC,suggesting that hypermethylation of the promoter region may be responsiblefor the silencing of these genes. In fact, methylation-specific PCR experiments showed that about 45% of tumors had hypermethylation of *p16^{INK4a}* and *p14^{ARF}* promoters. As expected, about 80% of human NMSC contained UV signature mutations in the *p53* gene and about 90% of the tumors were strongly positive for p53 immunostaining. Based onthese data, we conclude that in addition to mutations in the *p53* gene, silencing of *p16^{INK4a}*and *p14^{ARF}* gene expression via promoter hypermethylation plays an importantrole in the pathogenesis of human NMSC.

O11

Targeted Expression of bcl-2 to Murine Basal Keratinocytes results in Paradoxical Retardation of Chemical- and Ultraviolet B-induced tumorigenesis.

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In normal skin, the anti-apoptotic protein bcl-2, is present at low levels only in inter-follicular epidermis, but becomes highly expressed in several malignant and pre-malignant epidermal keratinocyte diseases. We have used transgenic mice that over-express human keratin-14 promoter-driven bcl-2 in the basal layer of epidermis to study the effect of deregulated bcl-2 expression on the incidence of UVB or chemically-induced epidermal tumors. Short-term UVB irradiation results in significantly fewer sunburn cells in the basal layer of the K14/bcl-2 mice than in controls, confirming the anti-apoptotic function of the transgene. However, K14/bcl-2, as well as K14/bcl-2xTG.AC mice, bearing an activated ras gene, were retarded (by 3 and 2 weeks respectively) in the time taken for 50% of mice to develop DMBA/PMA induced papillomas. Nevertheless, eventually all mice developed similar numbers of benign papillomas, and the conversion rate to carcinomas was similar in transgenic and control mice. UVB-induced carcinomas appeared with a latency of 23 weeks compared to 16 weeks in K14/bcl-2 mice and controls respectively, and significantly fewer transgenic mice developed carcinomas. Immunohistochemical analyses of the UVB-induced tumors revealed no significant differences in the degree of macrophage, neutrophil or T-cell infiltrates. We conclude that, despite its anti-apoptotic function, bcl-2, over-expressed in basal epidermal keratinocytes, exerts a paradoxical retardation on the development of skin tumors induced by chemical carcinogens, and particularly, by UVB.