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γδT Cells Regulate the Development of Hapten Specific Effector T Cells in Contact Hypersensitivity Responses

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γδT cells are widely located in the peripheral tissues and secondary lymphoid organs. It has been reported that γδT cells are required for transfer of contact hypersensitivity (CHS) responses by hapten primed T cells. However, the mechanism by which they do so remains to be elucidated. Initial experiments employed Tδ gene knockout mice that are deficient in γδT cells but are normal in the development of αβT cells. When mice were contact sensitized to DNFB, CHS responses were significantly greater in γδT cell deficient mice than in wild type mice. Similar results were obtained when wild type mice were depleted of γδT cells with antibody treatment before hapten sensitization. Depletion of CD4+ T cells did not affect the increased CHS response in γδT cell deficient mice, suggesting that the effect of γδT cells is on CD8+ T cells and does not require CD4+ T cells. Transfer of primed lymph node cells from hapten primed γδT cell deficient mice elicited a similar level of CHS in naïve wild type and the deficient recipient mice, indicating that γδT cells have little effects on the elicitation of primed T cells and CHS responses. Further experiments demonstrated that primed CD8+ T cells from the deficient mice exhibited significantly higher CTL activity than those from the wild type mice. The cytokine profile of CD4+ T cells was not significantly altered. We conclude that γδT cells down-regulate CHS responses to hapten sensitization by limiting the development of hapten specific CD8 + effector T cells during sensitization and that this effect is independent of CD4+ T cells.

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Expression of β-Defensins in Human Keratinocyte Cell Lines

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Defensins, a major family of antimicrobial peptides, are small cationic, cysteine-rich peptides with a wide range of antimicrobial activity. In human, β-defensin-1 was isolated from urine and cervical mucous suggesting that this peptide plays an antimicrobial role in the genitourinary tract. β-defensin-2 was identified in psoriatic scale produced by keratinocytes suggesting that this peptide contribute to defense the expansive surface of the integuments. This work was done to investigate the expression and modulation of β-defensin mrna in human keratinocyte cell lines. Cultured human keratinocytes were stimulated with ultraviolet B irradiation or tumor necrosis factor-α or lipopolysaccharide to determine whether defensin mrna production occurred. Reverse transcription polymerase chain reaction was performed to amplify defesin cDNA from stimulated keratinocytes, and Southern blots were used to verify the specificity of Reverse transcription polymerase chain reaction amplication products. Expression of human β-defensins was up-regulated with ultraviolet B irradiation, tumor necrosis factor-α and lipopolysaccharide in Hacat cells and in comparison to the control, significantly higher at 6 h post stimulation with ultraviolet B 100 mJ/§^{2} and peak at 12 through 18 h post stimulation with ultraviolet B 30 mJ/§^{2}, tumor necrosis factor-α and lipopolysaccharide. A431 cells did not show expression of human β-defensins in unstimulated state, even after stimulation with ultraviolet B irradiation or tumor necrosis factor-α or lipopolysaccharide. This report demonstrates the presence of defensin in the human keratinocyte and th capacity of human keratinocytes to produce defensin mRNA in response to ultraviolet B irradiation, tumor necrosis factor-α and lipopolysaccharide. Release of defensins by keratinocytes in response to cytokines elaborated in inflammation may contribute to the host defense response.

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Cathelicidin Antimicrobial Peptides Affect Adaptive Immunity by Regulation of Cytokine Production

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Cathelicidins are α-helical cationic peptides expressed by keratinocytes and granulocytes that are thought to be important mediators of innate immunity due to their direct antimicrobial action. To determine if their expression may influence adaptive immunity, cytokine responsiveness was measured in murine T cells, the Langerhans cell line XS52, and the human keratinocyte line HaCat after exposure to cathelicidins. Splenocytes or CD4-purified cells were stimulated with anti-CD3 and anti-CD28. At physiologic concentrations the murine cathelicidin CRAMP inhibited release of IL4 by 78% (1096 ± 249 vs. 241 ± 16 pg per ml), IL13 by 88% (2884 ± 874 vs. 357 ± 31 pg per ml), and IFNγ by 67% (7571 ± 1539 vs. 2464 ± 823 pg per ml). CRAMP did not affect IL6 production (368 ± 19 vs. 325 ± 26 pg per ml). Similarly, CRAMP inhibited IL1 β production from LPS stimulated XS52 cells (368 pg per ml ± 113 vs. 234 ± 15), though in the absence of LPS it stimulated IL1β release from undetectable baseline to 47.83 ± 6 pg per ml. The human cathelicidin LL-37 stimulated HaCat cells to produce IL1α (17 pg per ml ± 3 vs. 1.44 ± 0.79 at 5 h) and IL6 (524 pg per ml ± 14 vs. 308 ± 11 at 24 h). To determine if the decrease in cytokines was due to direct cytotoxic effects, viability and apoptosis was determined by propidium iodide (PI) staining and FACS analysis. CRAMP increased cell death in splenocytes (at 3 h, 19.5 ± 1.7 sd. vs. 35.4 ± 5.4%), and cellular debris with sub G0 DNA content (at 24 h, 12.6 ± 0.5 vs. 17.4 ± 1.7%). These results show that depending on the state of cell activation cathelicidins can inhibit or induce cytokines from T-cells, antigen presenting cells and keratinocytes. This suggests innate antimicrobial peptides may influence subsequent events in the adaptive immune response. Such an interation is a previously unknown event in cutaneous immunity that may play a role in several skin diseases.

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Analysis of the Regulation of Cathelicidin Antimicrobial Peptides in the Keratinocyte Line HaCaT

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Cathelicidins are a gene family of peptides that can directly kill bacteria while also inducing eukaryotic cell effects such as leukocyte chemo-attraction and synthesis of extracellular matrix components. Human cathelicidin (LL-37), is not detected in normal epidermis but is expressed in skin disorders such as psoriasis, contact dermatitis and wound healing. Similarly, murine cathelicidin (CRAMP) is induced in skin only when the normal skin is injured. Therefore, cathelicidin expression may provide antimicrobial resistance to the skin and be beneficial to wound repair. To investigate the mechanisms that lead to cathelicidin expression, we have stimulated HaCaT cells with known mediators of inflammation. Using quantitative RT-PCR by light-cycler, we find that the tumor promoter, phorbol myristate acetate (PMA), regulates the LL-37 expression in a time- and concentration-dependent manner. Human recombinant interferon-γ (IFN-γ) also modulates cathelicidin expression in a time-dependent fashion. In addition, we have detected LL-37 protein synthesis in response to IFN-? by dot blot analysis. Our preliminary data also implicate bacterial lipopolysaccharide (LPS) as a positive signal for induction of LL-37 expression in HaCat. To further study the regulation of cathelicidin expression, truncations of the 5′ flanking region of the murine cathelicidin CRAMP gene have been carried out. These constructs were cloned into a luciferase reporter system and the luciferase activity determined in a variety of cell types. Preliminary data indicate evolutionarily conserved regions of the CRAMP promoter that regulate gene expression. In conclusion, we show that cathelicidin expression can be modulated in response to inflammatory mediators. Since cathelicidins are versatile molecules with multiple functions in protecting the skin, these results offer a system that has potential applications for the therapy of human skin disorders.

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Altered Cutaneous Immune Parameters in Transgenic Mice Over-Expressing Viral IL-10 in the Epidermis

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IL-10 is a pleiotropic cytokine that inhibits several immune parameters including Th1 cell-mediated immune responses and cytokine production, antigen presentation, and antigen-specific T cell proliferation. In some systems, IL-10 augments Th2-type responses. Recent data implicate IL-10 as a mediator in the suppression of cell-mediated immunity induced by exposure to midrange ultraviolet radiation (UVB, 280–320 nm). Viral IL-10 (vIL-10) is ~80% identical with human IL-10 and also exhibits considerable sequence homology with mouse IL-10. To investigate the effects of IL-10 on the cutaneous immune system, we developed transgenic mice over-expressing vIL-10 in the epidermis. Epidermal cells from vIL-10 transgenic mice produced vIL-10 in culture and vIL-10 could be detected in serum. I-A-positive epidermal cells and dermal dendritic cells were reduced in number although the density of epidermal ATPase-positive cells appeared to be normal in epidermal sheets. After hapten painting of the skin, the number of I-A-positive, hapten-bearing cells detected in regional lymph nodes was reduced in vIL-10 transgenic mice compared to controls. CD80 and CD86 expression by I-A-positive epidermal cells was reduced in vIL-10 transgenic mice. After immunization with allogeneic cells, these mice demonstrated a significantly smaller delayed-type hypersensitivity (DTH) response to allogeneic cells upon challenge, but had normal contact hypersensitivity to an epicutaneously applied hapten. Fresh EC from vIL-10 transgenic mice showed a decreased ability to stimulate allogeneic T cell proliferation. Our data demonstrate the ability of vIL-10 to alter LC physiology and inhibit DTH. These findings support the concept that IL-10 is an important regulator of cutaneous immune function.

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Comparison of γ-Interferon Signal Transduction in Monolayer and Scaffold-Based Three-Dimensional Fibroblast Culture

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When fibroblasts in monolayer culture are treated with γ-interferon (IFN-γ), HLA-DR is induced and CD-40 is stimulated. By contrast, fibroblasts grown in three-dimensional culture on a scaffold show a major nonresponsive cell population. We have found that other IFN-γ-induced genes, including HLA class I and ICAM-1, show monotonic induction by all the cells in three-dimensional culture. We have investigated this system by immunoblot and by Taqman real time PCR to determine to what extent the IFN-γ-related signal transduction systems of the cells are affected by three-dimensional scaffold-based culture. Examination of STAT-1 phosphorylation by immunoblot has shown that this activity is essentially indistinguishable between monolayer and three-dimensional culture, indicating that the early stages of the IFN-γ signal transduction pathway are intact. We have examined the induction of the transcriptional regulator CIITA, which controls the expression of the HLA-DR region through interaction with DNA-binding proteins. Quantitative estimation of mRNA for CIITA has demonstrated it is induced in three-dimensional culture but only reaches a cellular concentration of a quarter that in monolayer. This may be accounted for by the minority of cells that shows induction. Thus, inhibition of transactivator expression is able to account for the lack of HLA-DR induction. In an examination of MAP kinases, we have found that p38 is not activated in three-dimensional culture as it is in monolayer. We conclude that the IFN-γ signal transduction systems is intact as far as STAT-1 phosphorylation, but inhibition of HLA-DR and CD40 induction is caused by lack of expression of CIITA. This effect may, in part, explain the lack of rejection of allogeneic fibroblasts cultured in this way.

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Dietary Soy Oil Content, and Soy Oil Derived Genistein Affect Susceptibility to Alopecia Areata in C3H/HeJ Mice

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Alopecia areata (AA) is a suspected autoimmune disease expressed in humans and rodent models. While oligogenetic susceptibility to AA is likely, epi-genetic factors may also affect AA expression. Diet as a susceptibility and severity modifier of AA in C3H/HeJ mice was examined. Prior to the experiment, all mice received a standard commercial diet of 1% soy oil (4% total fat). Normal haired C3H/HeJ mice were grafted with AA affected skin, a method previously shown to successfully induce AA. Grafted mice were given one of three diets. Twenty-eight mice continued to receive a 1% soy oil diet. Twenty-eight mice received a commercially available diet with 5% soy oil (7.5% total fat). Eleven mice received a diet with 20% soy oil (23% total fat) formulated by the animal diet manufacturer using the 1% soy oil diet as a base and additional soy oil from the same source. In a second study, 10 grafted mice were injected with 1 mg of the phytoestrogen genistein per week for 10 weeks and compared to 10 age matched mice receiving drug vehicle. Mice were monitored for at least 20 weeks after skin grafting and then necropsied. Twenty of 28 mice (71%) on the diet containing 1% soy oil developed AA hair loss by the time of necropsy whereas 11 of 28 mice (39%) on a 5% soy oil diet developed AA. Two of 11 mice (18%) fed a 20% soy oil diet had hair loss. Four of 10 mice injected with genistein and all 10 control mice developed AA. Mice that failed to develop AA typically regrew white hair from their skin grafts and histology revealed no apparent inflammation. Of those mice that expressed AA, no significant association was observed between the extent of hair loss and diet. Mice with AA had hair follicle inflammation consistent with observations for spontaneous mouse AA. The results suggest that soy oil may contain compounds with indirect or direct immunomodulatory properties that modify susceptibility to AA in mice and soy phytoestrogen genistein is a likely candidate.

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Characterizing C3H/HeJ Mice with Apparent Resistance to Alopecia Areata Onset

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Spontaneous alopecia areata (AA) develops in up to 20% of C3H/HeJ mice. The disease can also be induced in normal haired mice by grafting AA affected skin to their dorsal surface. Dietary Soy oil content and other environmental factors can reduce host susceptibility to AA onset after grafting. Mice that failed to develop AA after grafting were characterized by gross observation, adoptive transfer of lymphocytes, and FACS analysis of skin, lymph nodes and spleen cells. Sixteen mice that received AA affected skin grafts but failed to develop AA, received further AA affected skin grafts 15 or more weeks after the initial surgery of which 2 developed overt hair loss. In contrast, 14 of 19 control mice developed AA. Subcutaneous injection of 107 lymph node or spleen cells from grafted mice that failed to develop AA to 10 normal haired mice induced AA in 2. AA affected skin revealed an increased percentage of CD4+ , CD8+ and sIgM+ cells, an increase in IL-2, IL-4, IL-6, IL-10, IL-12, IFNγ and TNFα cytokine production, and strong up-regulation of the activation markers CD28, CD40L and their ligands CD80 (B7-1), CD86 (B7-2) and CD40. In skin draining lymph nodes high numbers of monocytes and dendritic cells were present and CD44v3, CD44v7 and CD44v10 were down regulated. In contrast, mice with failed AA induction had high numbers of monocytes and dendritic cells in the skin, increased expression of IL-2, IL-4, IL-10 and IFNγ, and down-regulated expression of CD95L and CTLA4. The results suggest that exposure to a variety of factors in AA affected skin grafts promotes disease onset or resistance to AA depending on the environment in which the stimulatory factors are presented. The costimulatory role of CD80, CD86 and their lymphocyte expressed ligands CD28 and CTLA4 may play a significant role in AA development. Examination of AA resistant mice may elucidate mechanisms of disease tolerance induction and will identify new therapeutic targets.

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Melanocyte Antingens Induce Expression of Mediators of Apoptosis in Melanocyte Specific T Cells in Vitiligo

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The immunological processes that mediate melanocyte destruction in vitiligo have recently been under renewed study. In this study, the role of melanocyte specific T cells and the expression of specific markers that mediate their response to melanocyte antigens were studied *in vitro*. Whole blood or isolated peripheral blood lymphocytes (PBL) from vitiligo patients and normal controls were exposed to melanocyte antigens for 18–96 h. Post activation cells were stained with flourecein labeled anti-CD3, CD69, CD28, CD95L, CD25, CLA, HLA-DR, and Annexin V. Flow cytometric analysis for activated cells, costimulatory molecules, skin homing receptor (CLA), apoptosis inducing molecules and apoptosis showed that 13 of 18 patients had increased expression of CD69 compared to controls. In this population, up-regulation of CD28 correlated with the presence of large number of cells with increased expression of MHC Class-II antigen, an indication that response to melanocyte antigens is a T cell receptor (TCR) mediated event. In addition, there was a 2-fold increase in CLA+ cells expressing CD95L. Annexin V stain revealed 0.5–2.7% increases in early apoptotic cells from vitiligo patients over normal control. These results suggest that destruction of melanocytes in susceptible patients may involve costimulatory, activation, and apoptotic molecules expressed by skin homing melanocyte-specific T cells.

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Osteopontin (OPN) Deficient Mice are Impaired in their Function to Attract Dendritic Cells to Lymph Nodes which Correlates with a Reduced Cutaneous Contact Hypersensitivity (CHS) Response

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OPN is a chemotactic protein that attracts inflammatory cells. The sensitization phase of cutaneous CHS is crucially dependent upon the migration of antigen-carrying Langerhans cells/dendritic cells (LC/DC) from the skin to draining lymph nodes (LN). Investigating the expression of OPN in the sensitization phase of CHS we found that OPN mRNA is up-regulated both in the TNCB hapten sensitized skin and in LN draining such skin after hapten application. Within skin especially endothelial cells expressed OPN. Speculating that OPN expression could be a crucial factor in attracting DC to lymphatic organs, we investigated the role of OPN in DC trafficking. Migration-assays revealed that OPN-induced DC migration in a chemotactic manner. *In vivo* s.c. injected OPN initiated LC emigration from the epidermis and OPN injected in close proximity to LN draining the skin attracted DC into these nodes. The OPN receptors CD44 and avβ3 integrin are known mediators of OPN induced cell migration. We found both receptors on DC upon their maturation in bone-marrow cultures. Antibodies against av and CD44 partially blocked OPN mediated LC/DC migration *in vitro* and *in vivo*. When ultimately investigating OPN function *in vivo* we found that OPN deficient mice had a significantly reduced CHS response to TNCB. Furthermore, when wild type DC were injected into OPN deficient mice, migration of DC to regional LN was reduced dramatically compared to wild type mice. In conclusion we demonstrate that OPN is a crucial factor in the initiation of CHS by guiding DC from skin into lymphatic organs.

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IL-4 Inhibits TNF-α-Induced Vascular Endothelial Growth Factor (VEGF) Production by Human Keratinocytes

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Vascular endothelial growth factor (VEGF) promotes endothelial cell growth, lymphatic vessels growth or leukocyte migration. Enhanced immunoreactivity of VEGF has been detected in the lesional skin of psoriasis, metastatic melanoma or Kaposi's sarcoma. We investigated the effects of various cytokines (IL-1β, TNF-α, IL-4, IL-6, IL-7, IL-8 and TGF-β) on VEGF production by human keratinocytes (KCs) cell lines, HaCaT cells *in vitro*. Both IL-1β (10 ng per ml) and TNF-α (10 ng per ml) significantly enhanced VEGF production for 48 h culture to 901.1 ± 102.2 pg per ml and 1526.0 ± 215.5 pg per ml, respectively. IL-4 (10 ng per ml) alone slightly inhibited VEGF production by KCs (22.3 ± 3.6%), but IL-6, IL-7, IL-8 or TGF-β did not significantly change this production. TNF-α (10 ng per ml)-induced VEGF production was significantly inhibited when HaCaT cells were cocultured with IL-4 (10 ng per ml) for 48 h to 37.4% (956.3 ± 140.3 pg per ml (IL-4 with TNF-α) v.s.1526.0 ± 248.0 pg per ml (TNF-α alone)). This inhibitory effect of IL-4 on TNF-α-induced VEGF production was observed at both dose and time dependent manner. RT-PCR and Northern blot analysis revealed that TNF-α-induced-VEGF mRNA was inhibited by IL-4 for 48 h culture, indicating that this inhibition occurred at mRNA levels. These data suggest that VEGF production by KCs can be modulated by Th2 cytokines and this suggest the possibility of future therapeutic approach for vascular proliferative disorders by selective cytokine such as IL-4.

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The Role of Reactive Oxygen Species in the Expression of Endothelial Adhesion Molecules in Allergic Inflammation

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In the pathogenesis of allergic diseases, such as atopic dermatitis, the expression of adhesion molecules and subsequent adhesion of inflammatory cells to endothelial cells is necessary. It is known that reactive oxygen species (ROS) are important second messengers in this process. We studied the effect of allergic reaction-related cytokines on production of ROS and the role of ROS in the expression of adhesion molecules in human dermal microvascular endothelial cells (HDMEC). ROS and adhesion molecules were determined by FACstar and ELISA, respectively, after stimulation with IL-1α, TNF-α, IL-4 and IL-13. Stimulation of HDMEC with IL-1α, TNF-α, IL-4 and IL-13 increased production of ROS. The expression of ICAM-1, VCAM-1 and E-selectin was up-regulated or induced by IL-1α, TNF-α and the expression of VCAM-1 was induced by IL-4, or IL-13. Stimulation of HDMEC with H2O2 up-regulated or induced the expression of ICAM-1, VCAM-1 and E-selectin but there was no synergistic effect between cytokines and H2O2. The expression of adhesion molecules up-regulated or induced by cytokines was inhibited by antioxidants. These findings suggest that ROS play an important role in the expression of ICAM-1, VCAM-1 and E-selectin induced by allergic reaction-related cytokines and pharmaceutical approaches manipulating reduction-oxidation mechanism would be a new therapeutic approach for allergic diseases.

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CCR4 Expression on Human Dermal Microvascular Endothelial Cells

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There is increasing evidence that the expression of certain chemokine receptors is not restricted to leukocytes, suggesting that they may play a role in processes others than leukocyte chemotaxis. Recently, it has been shown that certain chemokine receptors are expressed on endothelial cells. Little is known, however, about the expression of chemokine receptors on microvascular endothelial cells. In this study we show that the chemokine receptor CCR4, recently described as being associated with skin homing T cells, is expressed on human dermal microvascular endothelial cells (HDMEC). CCR4 expression in skin was demonstrated by immunohistochemistry. CCR4 immunostaining in normal skin and diseased skin (cutaneous T cell lymphoma) showed a constitutive CCR4 expression by dermal microvessels in normal skin and strong expression in lesional skin. Flow cytometric analysis confirmed CCR4 expression and showed that around 15% of cultured HDMEC are CCR4 positive. To gain more insight into the function of CCR4 on HDMEC, we examined the effect of the two CCR4 ligands, TARC (thymus and activation-regulated chemokine) and MDC (macrophage-derived chemokine) on the expression of adhesion molecules that mediate leukocyte adhesion to endothelial cells. We found that addition of TARC and MDC separately resulted in a twofold increase in baseline E-selectin expression on HDMEC. When TARC and MDC were added together a threefold increase was seen. In contrast, addition of TARC and MDC had no effect on the constitutive expression of ICAM-1 on HDMEC. These data suggest that release of TARC and MDC in the dermis may enhance the affinity of cutaneous postcapillary venules for CLA + T cells.

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The Neuropeptide α-MSH Acts as a Human Dermal Microvascular Endothelial Cell Survival Factor in Hypoxia

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Human dermal microvascular endothelial cells (HDMEC) play a key role in regulating cutaneous homeostasis and inflammatory responses in the skin. Hypoxic stress activates endothelial cells (EC) to release growth factors and pro-inflammatory mediators. In addition hypoxia may result in EC death, which contributes to ischemia and tissue injury. Little is currently known about the mechanism of EC death in response to hypoxia. α-MSH is a potent inhibitor of inflammation, and HDMEC are known to be both an α-MSH target and source of α-MSH. The plasma concentration of α-MSH is also raised during acute ischemia. Therefore, the aim of the present study was to test the ability of α-MSH to influence the survival of HDMEC under hypoxic conditions. HDMEC were cultivated under hypoxic conditions (0.5% O_2) for 24–36 h with increasing concentrations of α-MSH (10^{-14}–10^{-8} M). Cell viability was tested using a MTT based assay, EC were also stained for apoptotic bodies and the α-MSH precursor proopiomelanocortin (POMC) mRNA expression was determined by quantitative RT-PCR. Our results indicated that hypoxia significantly reduces cell viability and leads to the induction of apoptosis in HDMEC. In contrast, treatment with α-MSH prevented hypoxia-induced cell death in a concentration dependent manner, whereas TNF-α induced cell death is not affected by α-MSH. In addition, the expression of HDMEC POMC mRNA was markedly increased under hypoxic conditions. These data provide the first evidence that α-MSH, in addition to its anti-inflammatory capacity, may serve as a survival factor for HDMEC during hypoxic conditions and suggests that it could be used therapeutically in various cutaneous ischemic conditions such as vasculitis and chronic ulcers.

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E-Selectin is Constitutively Expressed by Dermal Blood Vessels: A Targeting Mechanism for Skin-Specific Immunosurveillance by CLA+ Memory T Cells

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The mechanisms regulating recruitment of memory T cells to skin are of central importance in immune surveillance for invading pathogens and developing malignancies. Induction of E-selectin (CD62E) on the vessel endothelium in response to inflammation permits tethering and rolling of skin-homing T cells expressing the Cutaneous Lymphocyte-associated Antigen (CLA), the required first step in leukocyte recruitment. While this process is well established for sites of cutaneous inflammation, the role of this pathway in immunosurveillance has been subject to controversy. Both E- and P-selectin are reported to be absent on routine immunohistochemistry of noninflamed tissues. We have shown previously, using intravital microscopy, that CLA+ human T cells and blood dendritic cells form spontaneous rolling interactions in the dermal postcapillary venules of unmanipulated mouse ears. This has been shown to reflect constitutive expression of selectins on mouse ear dermal microvessels. To determine if similar constitutive expression exists in human skin, we stained sections of normal uninflamed human skin for CD62E. Skin samples from three separate donors (face, breast and abdomen obtained from cosmetic surgical specimens) showed constitutive expression of E-selectin on a subset of vessels near the dermal-epidermal junction. None of these patients had clinical evidence of an inflammatory skin condition, nor did the skin samples show cellular infiltrates indicative of a subclinical inflammatory process. Serial sections stained with anti-CD31 showed numerous vessels throughout the sections that did not stain with anti-CD62E. Comparison sections from a patient with mycosis fungoides revealed numerous vessels staining with E-selectin within the tumor. Constitutive selectin expression in skin microvessels provides a mechanism for recruitment of CLA+ memory T cells to uninflammed skin and suggests an opportunity for selective inhibition or augmentation of normal and malignant T cell recirculation through skin in the therapy of inflammatory and malignant skin disorders

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Agonists of Protease-Activated Receptor-2 Induce Secretion of Interleukin-1β and Up-Regulation of Cell Adhesion Molecules in Human Dermal Microvascular Endothelial Cells

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Protease-activated receptor 2 (PAR2) belongs to a new subfamily of G protein-coupled receptors for serine proteases such as mast cell tryptase. Tryptase cleaves PAR2 and thereby induces cutaneous inflammation and infiltration of neutrophils. Recently we have shown that PAR2 plays a crucial role in cutaneous neurogenic inflammation. However, the precise role of PAR2-induced vascular responses during cutaneous inflammation is still uncertain. Therefore, we were interested in whether or not human dermal endothelial cells (HDMEC) express functional PAR-2 and whether agonists of PAR2 may regulate inflammatory responses in these cells. We detected expression of PAR2 in HDMEC cells by immunohistochemistry, FACS analysis and Northern blotting. Ca-mobilisation studies revealed that PAR-2 is functional in these cells. RT PCR showed up-regulation of IL-1β at 6 h after stimulation with tryptase and PAR2 agonist SLIGKV. ELISA experiments confirmed these results. We also investigated cell adhesion molecule expression after PAR2 activation by RT PCR and adhesion assay. ELAM-1 mRNA was up-regulated at 6 h and 9 h, VCAM-mRNA was up-regulated at 9 h, and ICAM-1 mRNA was up-regulated at 3 h after treatment with PAR2 agonists. In addition, cell adhesion assays demonstrated conclusive results. PAR-2 like immunoreactivity was increased in endothelial cells of patients with atopic dermatitis and urticaria indicating up-regulation of this receptor in endothelial cells during cutaneous inflammation. Electro mobility shift assays revealed PAR2-induced activation of NFκB. In conclusion, agonists of PAR-2 up-regulate IL-1β expression and release as well as expression of cell adhesion molecules in human dermal microvascular endothelial cells. Thus, PAR-2 may play an important role in cutaneous inflammation by mediating inflammatory responses of mast cell tryptase on endothelial cells and activation of NFκB.

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Expression of Toll Like Receptors in Human Keratinocytes

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Members of the Toll like receptor (TLR) family are involved in host defense and inflammation and are usually expressed by cells of the immune system such as macrophages. Since keratinocytes play a role in the skin immune response, we have determined if they may express the different members of the TLR family. The presence of mRNAs encoding TLRs was determined in cultured human keratinocytes by using RT-PCR. TLRs 1, 2, and 3 were strongly expressed, while TLR 4 was weakly detected. TLR 6 was undetectable. The positive expression of TLR2 in keratinocytes prompted us to assess the functionality of this receptor by treating cells with one of its ligand, a Gram + bacterial protein extract (SAC). After 8 h of treatment, we showed, by using an ELISA, that keratinocytes secrete IL12, a cytokine known to be also produced by macrophages after SAC treatment. Colonization of the skin surface with Staphylococcus aureus is a characteristic feature of patients with atopic dermatitis. We therefore studied the expression of TLR2 in pooled epidermis obtained from normal and atopic individuals. Expression of TLR2 was significantly decreased in disease as compared to normal epidermis. Our study showed, for the first time, the expression of Toll like receptors in human keratinocytes and that, after stimulation, TLR 2 is able to induce secretion of IL12. Furthermore, the differential expression of TLR 2 in the epidermis of patients with atopic dermatitis may indicate a role of this receptor in the pathogenesis of this disease.

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IL-18 Induces IgE Production in Caspase-1 Transgenic Mice and Wild Type Mice: Dependence on IL-4 and STAT6

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Overproduced IgE and Th2 cytokines are characteristic in atopic dermatitis and allergic disorders. Although IL-4 is critical to the polarization of CD4+ T cells to a Th2 phenotype, the epidermis derived factors that regulate IL-4 production and Th2 commitment *in vivo* are poorly understood. IL-18 that can be produced by keratinocytes and be processed by caspase-1, has strong ability in IFN-γ production from lymphocytes. Paradoxically, IL-18 has capacity to induce IL-4 and/or IL-13 secretion from NK cells, T cells and mast cells *in vitro*. To clarify the roles of IL-18 *in vivo*, we investigated skin-specific caspase-1 transgenic mice (CASP1Tg). CASP1Tg develop severe itching dermatitis with elevated serum IL-18 and IgE levels and the lesional IL-4 production. These responses were entirely suppressed in CASP1Tg lacking IL-4, STAT6 or IL-18. Furthermore, recombinant IL-18 injected into normal mice successfully elevated serum IgE levels. Results described above indicate that CASP1Tg is a potent model for atopic dermatitis, and caspase-1/IL-18 may be critically involved in regulation of IgE production *in vivo*, providing a potential therapeutic target for atopic dermatitis.

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In Vitro Method for Screening Anti-Irritants on UV and Chemically Treated Reconstituted Three Dimensional Human Skin

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A great need exists in the cosmetic, pharmaceutical and biochemical industries for *in vitro* methods to screen ingredients, to provide a quick and economical preclinical safety assessment, and to develop effective products. In the present study we utilized Epiderm(tm), a human skin equivalent culture, to test anti-irritants in a nonionic cream base. Treating Epiderm with known skin irritants such as UVA, UVB, SLS and croton oil resulted in a decrease in cell viability and an increase in the release of IL-1α. Cell viability (MTT) and IL-1α assays were conducted after application of the irritant or application of an anti-irritant followed by the irritant. A variety of tea extracts, rosemary extract,carnosic acid and rosemarinic acid (carnosic acid and rosemarinic acid are components of rosemary) showed protection of the cells against UVB, SLS and croton oil, although to different degrees. Rosemary extract reduced the release of IL-1α about 80% from the UVB treated Epiderm. However, carnosic and rosemarinic acids showed 50% inhibition of IL-1α. Green tea reduced 60% of IL-1 against UVB. Our results also suggest that green tea, rosemary extract, rosemarinic acid and carnosic acid are able to provide dose related protection against SLS and croton oil. These *in vitro* results showed good agreement with previously published and concurrent clinical tests using anti-irritants in the presence of known irritants. Our results demonstrate the usefulness of the living skin equivalent and of cell viability and IL-1α as biological markers for the screening of anti-irritants.

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Immunologic and Histopathologic Characterization of Active Disease Model Mouse for Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is an autoimmune blistering disease of skin and mucous membranes caused by antidesmoglein 3 (Dsg3) IgG autoantibodies. Recently, we have generated an active disease mouse model for PV by adoptive transfer of splenocytes from immunized Dsg3−/− mice to Rag2−/− mice. Recipient mice stably produced anti-Dsg3 IgG and inhibited adhesive function of Dsg3 with resultant blister formation. In this study, we performed immunologic and histopathologic characterization of this model mice and compared histologic features of PV model mice with those of Dsg3−/− mice. Direct immunofluorescence staining showed strong IgG as well as weak IgA deposition on cell surfaces of keratinocytes *in vivo* in PV model mice. Enzyme-linked immunosorbent assay revealed that IgG in PV mice specifically recognized mouse Dsg3, but not mouse Dsg1. The predominant subclass of anti-Dsg3 IgG was IgG1 both *in vivo* and *in vitro*. Suprabasilar acantholysis, typical histologic finding of PV, was similarly distributed in oral mucous membranes as well as on the skin around the eyes, nose, ears, limbs and tails in both PV model mice and Dsg3−/− mice. Acantholysis in telogen hair club caused patchy alopecia in both mice. However, microscopic blisters in esophagus and forestomach were only observed in PV model mice. Furthermore, some PV model mice developed severer phenotype with larger number of affected sites than Dsg3−/− mice. In addition, eosinophilic spongiosis, which is often found in PV patients, was occasionally observed only in the skin of PV model mice. These findings indicated that our PV model mice are valuable to investigate immunopathological mechanisms of blister formation as well as to evaluate various therapeutic strategies for PV.

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Search for T Cell Epitopes in Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is an autoimmune blistering skin disease mediated by autoantibodies directed against desmoglein 3 (Dsg3), a transmembrane protein involved in keratinocyte adhesion. Although tissue damage is ultimately mediated by anti-Dsg3 antibodies, an initial T-cell response is presumably required for autoantibody generation. The strong HLA association of PV to DRB1*0402 and DQB1*0503 reinforces the hypothesis that T-cell recognition of self and/or foreign peptides presented by self HLA molecules is a crucial initial step in disease development. A major focus of autoimmune research has been the precise identification of disease related T-cell epitopes. To define these epitopes in PV, we have designed a novel strategy which takes advantage of intracellular processing and presentation events to better capitulate natural ligands. Dsg3 has been targeted to the class II pathway of antigen presenting cells by transfecting chimeric vectors encoding intracellular trafficking signals. For this purpose, we have stably transfected the human melanoma cell line Mel JuSo (known to have a functional MHC class II antigen processing, presentation system) to express the disease susceptibility allele DRB1*0402. Additionally, we have constructed and transfected into DRB1*0402 expressing Mel JuSo cells 4 unique chimeric Dsg3 targeting vectors which utilize one of the following intracellular trafficking signals to ensure targeting to the appropriate intracellular compartments: (1) LAMP1 (lysosomal compartment), (2) LSYTRF (early endosome), (3) Mb (MIIC/late endosomal compartment), and (4) MSSSD (mutagenized-Mb tail targeting to the cell surface). Expression of these chimeric proteins in Mel JuSo cells has been confirmed by RT-PCR and Western blotting. Transfectant cell lines able to functionally stimulate T cell clones and PBMCs from PV patients will be grown in bulk in order to biochemically isolate MHC-peptide complexes by immunoaffinity column purification, followed by elution of bound peptides. Precise delineation of the presented epitopes will be confirmed by mass spectrometry and pooled peptide sequencing.

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The Distribution of IgG Subclasses in Pemphigus Vulgaris Patients in Active Disease and in Remission Ð Comparison of Direct Immunostaining (DIS), ELISA and Western Blot Data

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Several investigations on the role of pemphigus vulgaris pathogenic antibodies (PV-IgG) subclasses revealed a high frequency of apparently pathogenic IgG1 and IgG4 subclasses in patients' sera (Bhol *et al*, *PNAS* 92:5239, 1995; Kricheli *et al*, *Br J Dermatol* 143:337, 2000). The aim of this study was to determine whether their differential distribution in active and remission phases of the disease might further help in elucidation of the subclasses' pathogenicities. The study group comprised pairs of active/remission sera of 17 PV patients. The IgG subclasses profile was determined using direct immunostaining (DIS) with peroxidase-conjugated antihuman IgG of perilesional patients' skin, Dsg 3 ELISA (recombinant desmoglein 3) and Western blot (WB) reactivity with Dsg 3 in epidermal membrane extracts. Blood and skin biopsies were taken from the patients ín the same day. By ELISA/WB circulating PV-IgG 1 antibodies were found in the sera of 75% patients with active disease and in the sera of 33% of those in remission. In perilesional skin of PV patients in remission as well, the occurrence of tissue-fixed IgG1, determined by DIS, was lower (31%) as compared to their detection in patients with active disease (80%). On the other hand, circulating IgG4 antibodies were detected in sera of 80% patients with active PV and in sera of 50% patients in remission. By DIS, 100% of the patients with active PV had IgG4 fixed to perilesional epidermis, which was still detectable in 71% of patients in remission. In most of the cases when IgG was present in a remission counterpart of an active phase sample, it was with much reduced reactivity. Comparison of ELISA and WB data confirmed the sensitivity of the latter which, although reacting with denatured antigens, correlated in 90–100% of the cases to ELISA test which detects native antigens. The three techniques taken altogether showed a larger decrease in IgG1 occurrence (a drop of 57%) as compared to IgG4 (a drop of 33%) upon patients' remission. This might indicate the higher pathogenicity of IgG1 (a complement-fixing antibody). The significance of this finding is being further addressed to in our lab, by an ongoing study of *in vitro* pathogenicity of isolated IgG1 and IgG4 PV autoantibodies.

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Production of Human Monoclonal antibodies to Desmoglein 3 from Pemphigus Vulgaris patient

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Pemphigus vulgaris is a potentially fatal autoimmune mucocutaneous disease associated with production of IgG autoantibodies to desmoglein 3 (130-kDa protein). To further characterize the epitope (s) of pemphigus antigen we established two human-human hybridoma by fusion of the peripheral blood mononuclear cells with a human × mouse heterohybridoma. These hybridoma designated as RP06 and RP10 and stable in culture and demonstrated yield of monoclonal antibodies specific for pemphigus vulgaris. Immunofluorescence, immunoblot, ELSA assays demonstrated that both the monoclonal antibodies bind to the intercellular cement substance and to 130 kDa protein present in the epidermal lysate and specifically binds to recombinant desmoglein 3 protein, not to desmoglein 1 protein. The epitope mapping experiment using 12 peptides spanning the extracellular domain of pemphigus vulgaris antigen demonstrated that both the antibodies recognized the Bos 6 peptide and are of IgG1 subclass in nature. Both the monoclonal antibodies failed to produce acantholysis in normal human skin and unable to induce disease in neonatal BALB/c mice used in in-vitro and *in vivo* experiments, respectively. The relevance of these monoclonal antibodies in the pathogenesis of pemphigus vulgaris is discussed.

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Computer-Driven Individuation of Mouse Monoclonal Antidesmoglein 3 Antibody Defined Epitopic Peptide Sequence

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Utilizing computational biology, we have identified the antigenic linear determinant recognized by 5H10, a mouse monoclonal antibody (mAb) raised against the EC1/EC2 domains (aa1–212) of desmoglein-3 (Dsg3), the target of autoantibodies in pemphigus vulgaris. The computer-assisted search for the EC1/EC2 Dsg3 epitope was based on the analysis of (i) the capability of Dsg 3 15-mer peptides to bind to major histocompatibility complex (MHC) class II molecules and (ii) the similarity level of the human desmosomal adhesion protein to the mouse proteome. Among the peptides tested as possible antigenic determinants in dot immunoassay experiments, the anti-EC1/EC2 Dsg3 mAb 5H10 recognized the peptide having both high binding potential to MHC II molecules and low level of molecular mimicry to mouse proteome. These data validate previous results obtained in other disease models (breast cancer-associated-Her2/neu and cervical cancer-associated-HPV16 E7) and confirm the concept that peptide ability to bind to MHC molecules appears a condition necessary but not sufficient to determine peptide immunodominance, by needing to be supported by a low degree of similarity to the host's proteome.

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Isotype and Epitope Profiles of Anti-Desmoglein-1 Autoantibodies in Patients and Normal Subjects in an Endemic Focus of Fogo Selvagem

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Fogo selvagem (FS) is characterized by subcorneal blisters and antidesmoglein-1 (aDsg1) antibodies. Disease activity and remission correlate well with levels of IgG4 aDsg1 but not with levels of IgG1 aDsg1 antibodies (Warren *et al* Abstr.). IgG1 aDsg1 antibodies are detected in certain normal individuals and in preclinical stages of FS. The aim of this study was to investigate the epitope profiles of aDsg1 IgG isotypes present in FS (n:7) and control sera (n:8) using ELISA, immunofluorescence (IF), immunoblotting (IB) assays and monoclonal subclass-specific antihuman IgG antibodies. Recombinant Dsg1 (rDsg1) was used throughout for ELISA and IB studies and three tissue substrates, i.e. human (H), monkey (M) and mouse (Mus) for IF assays. The passive transfer mouse model of FS was also employed. All FS sera (n:7) tested contained IgG1 and IgG4 aDsg1 antibodies but normal control sera (n:8) contained only IgG1 aDsg1 antibodies by ELISA and IF analysis. IgG1 aDsg1 antibodies from FS sera bound epidermal cell surfaces from H, M, but not Mus by IF; whereas, control IgG1 aDsg1 bound only M esophagus. Further, FS IgG4 aDsg1 antibodies bound all 3 substrates by IF. IgG1 aDsg1 antibodies from patients (n:7) and controls (n:8) bound rDsg1 by IB; however, IgG4 aDsg1 antibodies were not reactive with rDsg1 by this assay. Affinity-purified aDsg1 antibodies from a control serum were ineffective in inducing skin disease in mice in a dose 10 times higher than those of pathogenic IgG4 aDsg1 antibodies. These results strongly suggest that IgG4 aDsg1 antibodies recognize a conformational epitope(s) on Dsg1 and IgG1 aDsg1 antibodies bind sequential and species-specific epitopes. The autoimmune response in FS may undergo epitope spreading during isotype switching from nonpathogenic IgG1 in the preclinical stages to pathogenic IgG4 when FS is clinically apparent.

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Analysis of T-Cell Receptor Vβ and Jβ usage in Pemphigus Foliaceus

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Analysis of T-cell receptors (TCR) is critical to further our understanding regarding mechanisms of T-cell epitope selection and autoimmune initiation and progression. Pemphigus foliaceus (PF) is an autoimmune skin disease characterized by intraepidermal blisters and circulating autoantibodies directed against desmoglein 1 (Dsg1), a 160 KD transmembrane desmosomal molecule expressed in keratinocytes. Although tissue damage is mediated by anti-Dsg1 antibodies, an initial T-cell response is a likely requirement for autoantibody generation in this disease. To further elucidate the role of pathogenic T-cells in autoimmunity, we have directly characterized the TCR of T-cells derived from PF patients. Complimentary DNA (cDNA) was isolated from 17 Dsg1 specific T-cell clones generated from PF patients by clonal expansion *in vitro*. To analyze the T-cell repertoire, a panel of primers, collectively specific for the known human TCR β variable region families were paired with a constant region primer to PCR amplify one distinct Vβ allele for each T-cell clone studied. PCR products were sequenced to determine exact β chain gene usage. Overall, 10 different Vβ gene segments were used. Five of 17 T-cell clones were found to utilize the same Vβ gene segment-Vβ5.1. However, no other particular Vβ subfamily was expressed more than twice. Nine different Jβ segments were utilized; Jβ2.5 was found in 4 of 16, and Jβ1.6 was found in 3 of 16 T-cell clones. While the selection of V and J gene segments was not randomly distributed, there was not exclusive usage of any single Vβ or Jβ segment. Instead, we demonstrate oligoclonality. Moreover, TCR gene usage varies within a given patient, indicating that disease induction and/or progression is not dependent on only one set of TCR gene segments.

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A Subset of Pemphigus Foliaceus Patients Possess Both Pathogenic Anti-Desmoglein-1 and Anti-Desmoglein-3 Antibodies

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Pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are distinct cutaneous autoimmune diseases characterized by intraepidermal blisters and antidesmoglein-1 (aDsg1) and antidesmoglein-3 (aDsg3) antibodies, respectively. PV patients exhibit aDsg3 antibodies, which in 50% of the patients are mixed with aDsg1 antibodies. We have shown that affinity-purified aDsg1 and aDsg3 antibodies from PV patients are pathogenic, inducing features of each disease in mice. Moreover, clinical and serological transition from PV to PF has been reported in few patients. The majority of PF patients; however, show mainly aDsg1 antibodies. A recent report describes a transition from PF to PV in a single patient. The aim of this study was twofold: (a) to investigate the presence of aDsg1 and aDsg3 in a large group of PF patients (n:276) by a highly sensitive ELISA and immunoprecipitation (IP) assays and (b) to test the pathogenicity of aDsg1 and aDsg3 antibodies in the mouse model. Purified soluble fractions comprising either the ectodomain of rDsg1 or rDsg3 were generated in the baculovirus system and used in the assays. We detected aDsg1 antibodies in all PF sera tested (100%) and aDsg3 antibodies in 19 PF sera (7%) using ELISA and IP tests. To further verify the specificity of both autoantibodies, an inhibition ELISA was employed using known amounts of purified rDsg1 and rDsg3. The rDsg3 was able to inhibit the binding of only aDsg3 antibodies in a dose-dependent manner, whereas rDsg1 blocked only the binding of aDsg1 in a dose-dependent manner. Passive trasfer experiments of affinity-purified aDsg1 antibodies from 3 PF patients induced subcorneal acantholysis in mice and aDsg3 antibodies induced suprabasilar acantholysis in these animals. These studies suggest that in a subset of PF patients there is a dual and independent pathogenic autoantibody response against Dsg3 and Dsg1.

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The Role of IgG Subclass Switch in the Pathogenesis of Fogo Selvagem

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Fogo Selvagem (FS) is characterized by subcorneal blisters and antidesmoglein 1 (aDsg1) antibodies. The endemic focus of FS in Limao Verde (LV), Brazil shows a disease prevalence of 3.4%. In LV, we found aDsg1 antibodies in 97% of the FS (n:31) and in 55% of normal subjects (n:93). In this study, we have determined the IgG subclass aDsg1 response in patients (n:31) [preclinical (n:5) and remission stages (n:8)] and normal subjects known to have high levels of aDsg1 antibodies (n:31). We detected IgG1 aDsg1 antibodies in 25 of 31 patients (mean ELISA value 4.8), and IgG4 aDsg1 antibodies in 24 out of 31 (mean ELISA value 112.53). The 31 normal subjects showed a mean value of IgG1 aDsg1 antibodies of 4.74; this value was very close to the mean value of IgG1 found in FS patients. However, the mean value of IgG4 aDsg1 antibodies in this normal group was 5.82, which is 19.3 fold lower than in the FS group. IgG1 aDsg1 antibodies were detectable 1–4 years before the onset of FS in 5 patients. Onset of clinical FS in this group was associated with a mean 1.95 fold increase in IgG1 aDsg1 antibody and a 9.54 fold increase in IgG4 aDsg1 antibody levels. Finally, the level of IgG4 aDsg1 antibodies also correlated closely with activity and remission of FS. A positive IgG4 aDsg1 antibody test was associated with active disease in 22/23 patients, whereas a negative IgG4 aDsg1 antibody test was associated with clinical remission in 7/8. These correlations were not observed with the level of IgG1. These results suggest that progression of FS from preclinical to clinical disease is associated with a switch from IgG1 aDsg1 antibody to an IgG4 autoantibody response. IgG1 aDsg1 antibodies may not be pathogenic.

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Serum IgG from Pemphigus Foliaceus Patients Reacts Against Maxadilan

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Endemic Pemphigus Foliaceus (EPF) is an autoimmune skin disease caused by autoantibodies against desmoglein 1, the transmembrane glycoprotein of desmosomes. Vieira reported in the 1940s that two thirds of the State of São Paulo was affected by EPF, with the highest incidence of disease in the north-east region, from Franca to Ribeirão Preto, extending to the margins of the Grande River. He noted that the Simulium fly (black fly, ''borrachudo'' in Portuguese) was common in the areas most affected by EPF. Maxadilan is a potent vasodilator peptide present in salivary glands of the sand fly Lutzomyia longipalpis, a vector of leishmaniasis. In that region, pemphigus foliaceus and cutaneous leishmaniasis are endemic. The purpose of this study was to determine if serum antibodies against maxadilan are present in EPF patients. Serum obtained from a total of 38 people from this region including 23 patients with EPF, 4 with pemphigoid and 11 controls were incubated with maxadilan [143 μg per ml] followed by incubation with human anti-IgG in an ELISA test. Optical densitometry was obtained at 492 nm in a spectrophotometer. The results with EPF serum varied from 0.060 to 0.804; of pemphigoid serum from 0.039 to 0.282, and of control serum from 0.060 to 0.219, with significance, $p < 0.01$, between EPF and control groups. There was no statistical difference between localized (median, 0.360) and generalized (median, 0.522) forms of EPF. There was no correlation ($r = -0.2414$) between serum IgG antimaxadilan (ELISA, median 0.304) and serum IgG antidesmoglein (indirect immunofluorescence, median 1:1024) in 16 EPF samples. These results demonstrate that patients with EPF have antibodies against maxadilan. The relationship between the antibodies to maxadilan and the autoantibodies that react against desmoglein 1 in HLA susceptible individuals for EPF needs to be clarified.

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IgG, IgG1, IgG4 and IgE Antibodies to Three Domains of a 230-kDa Bullous Pemphigoid (BP) Antigen in BP Sera

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It is known that IgG1 and IgG4 subclasses of IgG autoantibodies predominate in bullous pemphigoid (BP) sera. It has also been reported that IgE antibodies target both 230 kDa (BP230) and 180 kDa BP antigens. The aim of this study was to examine BP sera for the presence of IgG, IgG1, IgG4 and IgE antibodies to the three bacterial recombinant GST-fusion proteins of C-terminal (BP230-C), middle (BP230-M), and N-terminal (BP230-N) domains of BP230, which encompass the entire molecule of BP230. Sixteen sera from BP patients showing typical immunological features of BP, 1 pemphigus vulgaris serum and 3 linear IgA bullous dermatosis sera were examined by immunoblotting. There were 8, 3, 6 and 2 BP sera with IgG, IgG1, IgG4 and IgE antibodies, respectively, reactive with BP230-C. There were 6, 4, 6 and 2 BP sera with IgG, IgG1, IgG4 and IgE antibodies, respectively, reactive with BP230-N. There were 2 BP sera with IgG, but not with IgG1, IgG4 or IgE antibodies, reactive with BP230-M. None of the control sera had antibodies of any class/subclass to any of the recombinant proteins of BP230. These results suggest that the production of IgG4 and IgE antibodies to BP230 might not be significantly correlated in patients with BP at blistering stage of their disease.

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A Transgenic Approach For Model Immune Responses Against Bullous Pemphigoid Antigen 2 (BPAG2)

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BPAG2, a 180-kDa, type II transmembrane protein associated with hemidesmosomes in basal keratinocytes, is targeted by autoantibodies in patients with various blistering diseases. To elucidate the primary pathophysiology of these autoimmune diseases, C57BL/6 transgenic (Tg) mice expressing human BPAG2 in murine epidermis under the control of a human keratin 14 promotor were produced. Four phenotypically normal founders were identified and bred. Monoclonal and polyclonal anti-BPAG2 IgG identified human BPAG2 in murine epidermal basement membranes (BMs) in 3 of 4 founder lines; expression was greatest in mice homozygous for the transgene. Grafts of trunk or tail Tg skin placed on otherwise syngeneic C57BL/6 mice (wt) were lost in 3 weeks; wt mice grafted a second time with Tg skin showed graft loss within 1 week. Sera from mice grafted with skin from 2 different Tg founder lines contained IgG that: (1) bound epidermal BM in human and Tg skin; (2) immunoblotted human BPAG2; and (3) showed no reactivity to epidermal BM or BPAG2 in wt C57BL/6 mice. Immune splenocytes of grafted wt mice generated B-cell hybridomas producing monoclonal IgGs that specifically bound human epidermal BM and BPAG2. Adoptive transfer of immune splenocytes from grafted wt mice to naive Tg mice expressing human BPAG2 sustained production of circulating anti-BM IgG *in vivo* for more than 28 days (the longest period of observation to date). This transgenic approach yielded an animal model in which primary immune responses against a known autoantigen can be elicited, characterized, and potentially modulated or blocked *in vivo*. This animal model may elucidate the primary pathophysiology of autoimmune blistering diseases.

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Development of an Active Autoimmune Disease Model for Bullous Pemphigoid

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Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease characterized by deposition of IgG and complement components at the basement membrane zone (BMZ) and inflammatory infiltration at the dermis. BP autoantibodies recognize two hemidesmosomal proteins – BP230 and BP180. The current IgG passive transfer animal model for BP reproduces key immunohistological features of human BP. We have used this model to systematically dissect the inflammatory cascade and demonstrate that subepidermal blistering is triggered by anti-BP180 IgG and depends on complement activation, mast cell degranulation and neutrophil infiltration. Activated neutrophils release proteolytic enzymes and reactive oxygen species, causing subepidermal blistering. To understand the molecular and cellular interactions between T and B lymphocytes leading to the production of pathogenic anti-BP180 IgG, we have attempted to develop an BP model by active immunization of mice with murine BP180 antigen. Three strains of mice BALB/c (H2d), C57BL/6 J (H2b), and SJL/J (H2s) were immunized with recombinant mBP180 antigen (mBP180ABC) and compared for BP-susceptibility and immune-responsiveness to the autoantigen. Although all three strains of mice produced antimBP180 IgG as determined by immunoblotting, ELISA and indirect immunofluorescence, only C57BL/6 J mice are susceptible to BP. We found that approximately 20% immunized mice developed skin lesions. Histological examination of the lesional sites in these mice revealed a BP-like dermal-epidermal separation with inflammatory cell infiltration. *In vivo* deposition of IgG and C3 was found at BMZ. Further, about 80% immunized C57BL/6 J produced pathogenic anti-BP180 IgG as determined by passive transfer experiments. This model will allow us to investigate T and B-cell responses that modulate BP and develop novel therapeutic strategies for this disease.

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IgG Auto-Antibodies to BP180 are Directly Pathogenic in Human Skin and Induce Sub-Epidermal Blisters: Evidence Using Skin Explant Culture and SCID Mouse Grafted with Human Skin

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Previous studies had suggested that antibodies to the 180 kDa bullous pemphigoid (BP180) antigen can induce subepidermal blisters only in the presence of complement and PMN/eosinophils. We here provide evidence that anti-BP180 antibodies are pathogenic and induce subepidermal splits without presence of complement or PMN/eosinophils *in vitro* in a skin explant culture model and *in vivo* in SCID mouse grafted with normal human skin. IgG4 deposits were found along the dermal-epidermal junction and localized to the epidermal side of the split. C3 deposits were insignificant and there were no major eosinophilic infiltrates in the split area. Clinically, a correlation was found between the presence of IgG4 antibodies to BP180 and disease severity in 20 patients with bullous pemphigoid (BP) ($p < 0.001$). Sera from patients with severe forms of the disease contained high titer anti-BP180 antibodies. We further sought to determine the mechanism by which the anti-BP180 antibodies may mediate their pathogenic effect by using a keratinocyte culture system and a rabbit antibody raised against the extracellular part of BP180. Pre-incubation of cultured keratinocytes with the anti-BP180 antibody induced re-distribution of BP180 ectodomains from the cell membrane into the cytoplasm. This change was accompanied by BP180 phosphorylation. The present study demonstrates the direct pathogenic role of anti-BP180 antibodies in human skin in both *in vitro* and *in vivo* models.

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Detection of BP180 Specific IgE Antibodies in Bullous Pemphigoid and Identification of a Potential Effector Function

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Bullous pemphigoid (BP) is an autoimmune blistering disease characterized by the presence of autoantibodies directed against two hemidesmosomal proteins in the skin: BP180 and BP230. Animal models have provided strong evidence that IgG antibodies reactive with the NC16A domain of BP180 play a direct role in disease pathogenesis. Recent studies have indicated that anti-BP180 IgE class antibodies are also present in BP sera; however, the pathologic relevance of the IgE response has not been established. The present studies were designed to further characterize the role of IgE antibodies in BP. Seventeen BP patients well characterized by clinical, histological, and immunological criteria were used in these studies. By immunoblotting, IgE reactivity to NC16A was detected in 6 of 7 (86%) new, untreated BP patients and in an additional 5 of 10 BP patients at various stages of treatment. By indirect IF 5/17 sera contained IgE antibodies that bound the basement membrane zone (BMZ). Of the 13 patients' skin biopsies that were available for testing, none had detectable IgE bound to the BMZ *in vivo*; however, 9 of the 13 samples showed IgE-positive cells in the papillary and reticular dermis. These IgE-positive cells were identified as mast cells by double labeling with Texas-Red conjugated antihuman mast cell tryptase. Basophils from freshly drawn blood of 5 BP patients were challenged with varying amounts of recombinant NC16A and the release of histamine was measured using a competitive histamine immunoassay. Cells from 4 of the 5 BP patients exhibited NC16A-dependent histamine release. The one patient that showed no response was in remission on steroids and dapsone. Basophils from 6 controls were assayed and none showed a response to NC16A. These data suggest that IgE class antibodies may have a role in the pathogenesis of BP.

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Antibody to an Intracellular Domain of Human β4 Integrin is Pathogenic in Mucous Membrane Pemphigoid

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Mucous membrane pemphigoid (MMP) is a mucocutaneous autoimmune blistering disease characterized by the presence of an autoantibody to the basement membrane zone (BMZ). We identified human β4 to be the target antigen recognized by these anti-BMZ antibodies. We have previously demonstrated that MMP autoantibody reacts with the intracellular and not the extracellular domain of human β4 integrin molecule. We produced 3 essential fragments of the intracellular region of β4 (IC1.0, IC2.0 and IC3.0). Majority of the MMP sera demonstrated strong binding to IC3.0. The binding of the MMP sera to IC3.0 is entirely due to its binding to IC3.4 containing 85 aa. This region may have multiple antibodies binding sites. We incubated MDA-435 cell line (human β4 expressing cell line) with rabbit antibody to IC 1.0, IC2.0, IC3.0, IC3.4, MMP sera, normal human sera and preimmune rabbit sera. Using immunoperoxidase staining binding of MMP sera, and rabbit antibodies to different intracellular fragments of β4 integrin to the cytoplasm of MDA-435 cells was observed. No binding of preimmune rabbit sera or normal human sera to the cell lines was observed. These experiments clearly indicate that these antibodies can penetrate the cells and bind to relevant antigens. Rabbit antibody to IC3.0 and IC3.4 produced separation of BMZ when incubated with normal human buccal mucosa in an *in vitro* organ culture model. Similar BMZ separation of oral mucous membrane is observed in cultures incubated with MMP patient sera and antibodies to ?4 integrin, but not with normal human sera. These observations identify IC3.4 or CP1 as the possible antibody binding site for sera of MMP patients and suggests a possible role for it in blister formation. The observations in this study provide a model that allows the use of normal human buccal mucosa in studying the pathogenesis of MMP, blister formation, hemidesmosomal and basement membrane biology.

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Immunomapping of EBA Sera to the NC-1 Domain of Collagen VII: Further Evidence that Anchoring Fibrils Originate and Terminate in the Lamina Densa

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Epidermolysis bullosa acquisita (EBA) is an autoimmune blistering disease with circulating antibodies to type VII collagen, a major component of anchoring fibrils located in the epidermal basement membrane. We assessed the ultrastructural localization of sera from two Japanese patients with clinically typical forms of EBA and compared this with two EBA patients who predominantly presented with oral lesions initially and mild cutaneous involvement. Immunoblotting of whole dermal extracts showed labelling of a 290-kDa target in both typical and mucosal EBA forms, consistent with that of type VII collagen. The blotting target for the EBA sera was further identified using the NC-1 domain fusion protein of collagen VII and the central collagenous portion of collagen VII. Postembedding immunoelectron microscopy (IEM) using Lowicryl K11M embedded normal human skin and patient's sera demonstrated the majority (80%) of labelling over the lamina densa, not below the lamina densa. This labelling pattern was similar to that observed when using the mouse monoclonal LH7 : 2 against the NC-1 domain of collagen VII but was distinct from the NC-2 domain labelling. The majority of EBA sera blotted to the NC-1 domain (3/4 sera). One typical EBA serum reacted with both NC-1 and the collagen VII triple helical domain, confirming the latter as a less frequent binding epitope. We conclude that the majority of EBA sera bind to the NC-1 domain of collagen VII situated in the lamina densa of the epidermal basement membrane. This further confirms previous reports that anchoring fibrils originate and terminate in the lamina densa. The presence of multiple EBA epitopes on the collagen VII molecule shows no correlation with disease phenotype and therefore an individual's immune response is more likely to determine the clinical features of this disease.

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The α5 and α6 Chains of Type IV Collagen are the Target of Autoantibodies in a Novel Autoimmune Disease Characterized by Subepidermal Blisters and Glomerulonephritis

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Type IV collagen is a heterotrimeric complex of proteins composed of α1(IV)-α6(IV) chains. The α5(IV) and α6(IV) chains are expressed at the dermal-epidermal junction, but their precise function is as yet unknown. We here describe a novel autoimmune disease with IgG or IgA autoantibodies directed against the NC1 domain of the α5(IV) or α6(IV) type IV collagen chain. Clinically, the patients presented with severe subepidermal bullous eruptions and glomerulonephritis. *In vivo* deposits of IgG or IgA were found along the dermal-epidermal junction and glomerular basement membrane zone. The identity of the target antigen was determined by immunochemical analyses of candidate antigens using the patients' autoantibodies. These autoantibodies reacted with a 185–190 kDa band from human dermal extracts that was distinguished from the known 180 kDa or 230 kDa bullous pemphigoid antigens, the 290 kDa (type VII collagen) epidermolysis bullosa antigen or the α3, β3 and γ2 chains of laminin 5 cicatricial pemphigoid antigen. Affinity purified IgG and IgA antibodies from the 185–190 kDa nitrocellulose band specifically reacted with recombinant NC1 domain of α5(IV) or α6(IV), among the six NC1 domains of type IV collagen, by ELISA. This study provides evidence for the α5(IV) and α6(IV) chains as target antigens in a novel autoimmune disease characterized by severe skin blistering and glomerulonephritis.

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IQGA Human Ras GTPase-Activating-Like Protein IQGAP1 is Expressed by Human Keratinocytes and Recognized by Circulating Autoantibodies

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Autoantibodies in patients with bullous skin diseases are of diagnostic value and suspected to play a role in the initial pathogenic scenario. We screened serum samples from patients with bullous pemphigoid, pemphigus, erythema multiforme and erythema multiforme like bullous eruptions for the presence of circulating autoantibodies. Immunoblotting and immunoprecipitation of lysates from cultured human keratinocytes revealed a sofar unrecognized distinct protein band of 190 kDa in a small number of patients (4 out of 120). For characterization aminoacid sequence analysis of peptide fragments derived from the respective protein after preparative SDS-gel electrophoresis was performed showing 100% sequence homology to IQGAP1 (IQGA Ras GTPase activating-like protein), a recently described protein suspected to act as an effector molecule for Cdc42 and Rac1, members of the Rho small GTPase family and to play a key role in regulating E-cadherin mediated cell-cell adhesion. The protein is also selectively recognized by a bonified anti-IQGAP1 monoclonal antibody on Western blots and immunoprecipitates from cultured human keratinocytes and epidermal extracts. Indirect immunofluorescence locates IQGAP1 within individual keratinocytes in a perinuclear, vesicular pattern and in the periphery of the cell close to the inner part of the cell membrane. In conclusion, our results demonstrate that IQGAP1, a newly described multifunctional protein, is constitutively expressed in human keratinocytes where it may contribute to the integrity of the epidermal layer. At the same time IQGAP1 appears as target for circulating autoantibodies in a small number of patients exhibiting erythema multiforme like eruptions.

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NPY Suppresses Induction of Contact Hypersensitivity

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An anatomical association between epidermal Langerhans cells (LC) and peripheral nerves has recently been discovered. It has been hypothesized that the nervous system regulates the function of LC via release of neuron-derived factors, resulting in modulation of cutaneous immune responses. Neuropeptide Y (NPY) and peptide YY (PYY) are structurally related peptides with a variety of known functions, including regulation of cardiovascular tone and and food/water-intake. NPY has been found in nerve endings in immunocompetent organs, including the epidermis. Several authors have described an effect of NPY and PYY on immunocompetent cells, suggesting a role for these neuropeptides in immune functions. In this study we wished to determine whether NPY and PYY affect the antigen presenting capability of murine LC. We utilized a contact hypersensitivity (CHS) model in which CAF1 (BALB/c × A/J) F1 (H-2d/a) mice (female, 8–10 weeks) were injected i.d. with 500 pmol of NPY or PYY or diluent alone 15 min prior to sensitization with 5 ml of 1% DNFB at the same site. Seven days later mice were challenged by application of 5 ml of 0.2% DNFB to both sides of their ears and 24 h ear-swelling was assessed. Injection of NPY significantly reduced CHS by 73% ($p < 0.01$) (NPY injected group: 2.9 mm-2 vs. positive control/no NPY: 10.65 mm-2; negative controls received vehicle without DNFB before injection of NPY or diluent: 0.2 and 0.04 mm-2, resp.; data pooled from two independent experiments, $n = 10$). In contrast, PYY and substance p had no significant effect on the DNFB-induced ear swelling response. We wished to determine whether the above findings could be explained by a direct effect of NPY on LC. However, RT-PCR failed to demonstrate NPY 1-, 2- or 5-receptors, specific for NPY and PYY, on fresh murine LC and the LC-cell lines XS52 and XS106. Thus, NPY might not affect LC antigen presenting capability and function directly. We can infer, however, that the observed effect of NPY is unrelated to its known vasoconstrictive function, since PYY, equipotent as a vasoconstrictor, did not affect CHS-induction at the same dose in this model. We plan to further elucidate the exact mechanisms involved (e.g. stimulation/inhibition of T-cell and/or keratinocyte cytokine production) in future experiments.

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Repeated Hapten Application Induces Th1 Deviation in NC/Jic Mice (Subspecies of NC Atopic Model Mice)

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NC/Nga mice show atopic dermatitis-like eruption and increase in serum IgE in conventional conditions but not in specific pathogen-free (SPF) conditions. It has been reported that NC/Nga mice also show a high serum IgE level and dermatitis after repeated trinitrochlolobenzene (TNCB) application in SPF conditions. We applied TNCB to another NC mice strain, NC/Jic, once a week for 4 weeks in SPF conditions. After the last application of TNCB, we studied immune reactions, including serum IgE level, delayed type hypersensitivity (DTH) reaction and cytokine profile in regional lymph node. Non-treated NC/Jic mice showed a serum IgE level higher than those of the control BALB/c mice in coventional conditions, but after the application of TNCB, the NC/Jic mice showed more severe dermatitis, lower serum IgE level and higher serum IgG2a level than did the control BALB/c mice in SPF conditions. Moreover, NC/Jic mice showed stronger DTH reaction, higher IFN-γ level and lower IL-4 level in regional lymph nodes compared to control BALB/c mice in SPF conditions. The results indicate that the NC/Jic mice showed a Th2 type response in conventional conditions but a Th1 type response after repeated hapten application in SPF conditions compared to control BALB/c mice. It is thought that this difference in T cell deviation in NC/Jic mice may be due to antigens or antigen routes.

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Th2 Memory in a Mouse Allergic Asthma Model

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Immunological memory is one of the features of immune system to react more rapidly and more effectively to the second exposure of antigen. Whereas life long protection against pathogen is beneficial vigorous and long immune responses against allergen is not. Several studies examined the CD8 and Th1 memory but the Th2 memory is not well understood. To study the Th2 memory response we generate a mouse allergic asthma model in the absence of adjuvant and microorganisms. BALB/c female mice were immunized intraperitoneally with either 10 μg of ovalbumin (OVA) or PBS on days 0 and 21, 7 days later they were nebulized with 1% OVA dissolved in PBS or PBS alone twice a day for 2 days. To study the generation and maintenance of Th2 memory in this mouse allergic asthma model OVA sensitized mice were nebulized with OVA or PBS and mice were assessed on day 60, 90, 143, 170, 220, 260 and 433. Mice, which were aerosol challenged with OVA showed BAL Eosinophilia and airway hyperresponsiveness, high level of mucus production and peribronchial and perivascular infiltration, high level of IL5 production less amount of IL4 whereas OVA sensitized mice which were aerosol challenged with PBS showed no Eosinophilia and airway hyperresponsiveness and no mucus production but they show peribronchial lymphocytes infiltration. Splenocytes from PBS challenged mice are producing lower amount of IL4 and IL5 compared to OVA challenged mice. In summary, we generate a mouse allergic asthma model in the absent of adjuvant and microorganisms to study a th2 memory in this model. Th2 memory cells are generated and maintained until 400 days after sensitization.

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CC Chemokine Receptor (CCR4) Expression on Peripheral Blood CD4+ T Cells Reflects Disease Activity of Atopic Dermatitis

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Th1 and Th2 cells differ in their chemokine receptor expression and their responsiveness to various chemokines. CC chemokine receptor (CCR) 4 is selectively expressed on Th2 cells, whereas CXC chemokine receptor (CXCR) 3 is selectively expressed on Th1 cells. We examined CCR4 and CXCR3 expression on peripheral blood CD4+ and CD8+ T cells in atopic dermatitis (AD) ($n = 17$), psoriasis vulgaris (Ps) ($n = 9$) and healthy subjects ($n = 9$). CCR4 expression on CD4+ T cells from AD subjects (22.4 ± 18.1%) was significantly higher than that in healthy subjects (5.3 ± 3.5%, $p < 0.005$) and Ps subjects (6.5 ± 5.0%, $p < 0.05$). CXCR3 expression on CD4+ T cells in AD, Ps and healthy subjects were 9.1 ± 5.1%, 25.6 ± 8.5% and 9.2 ± 5.5%, respectively. Intracytoplasmic IL-4 or IFN-γ staining in CCR4+ or CXCR3+CD4+ T cells of AD subjects after PMA and ionomycin stimulation revealed that 43.4 ± 7.5% of CCR4+CD4+ T cells were positive for IL-4, but virtually negative for IFN-γ and that 29.4 ± 5.4% of these cells were positive for both IL-4 and IFN-γ, suggesting that most of these cells were Th2 or Th0 cells. CCR4 expression on CD4+ T cells in severe AD subjects decreased by improvement of disease activity. CD25 was preferentially expressed on CCR4+CD4+ T cells (20.1 ± 7.2%) but not CXCR3+CD4+ T cells (6.7 ± 1.7%) in AD subjects. Cutaneous lymphocyte-associated antigen (CLA) was preferentially expressed on CCR4+CD4+ T cells (24.9 ± 8.4%) but not CXCR3+CD4+ T cells (1.3 ± 0.8%) in AD subjects. Immunohistochemical analysis indicated that CD4+ T cells in AD skin lesion were predominantly CCR4+ cells. These data strongly suggest that CCR4 expression is important for T cell infiltration into AD lesions.

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Thymus and Activation-Regulated Chemokine (TARC) in Atopic Dermatitis: Serum Level of TARC is Closely Related with Disease Activity

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Thymus and activation-regulated chemokine (TARC/CCL17) is a chemokine that attracts CC-chemokine receptor 4 positive (CCR4+) or CCR8+ cells. We have previously demonstrated that TARC is produced by epidermal keratinocytes (KCs) using NC/Nga mouse, regarded as a mouse model of atopic dermatitis (AD) (*J Clin Invest* 104:1097, 1999). In order to investigate the participation of TARC in AD, we examined the serum TARC levels of AD (n = 40), healthy controls (n = 20) and psoriasis vulgaris (Ps) (n = 20). The serum TARC levels of AD patients (2338.70 ± 302.83 pg per ml) were significantly higher than those of healthy controls (215.3 ± 26.79 pg per ml) and Ps patients (256.30 ± 113.10 pg per ml). The serum TARC levels of AD decreased after the treatment in accordance with the improvement of clinical symptoms. The serum TARC levels in AD significantly correlated with eosinophil number (coefficient:$r = 0.61$), serum sE-selectin levels ($r = 0.58$) and weakly correlated with serum sIL-2R levels ($r = 0.34$). CCR4 positivity of CD4+CD45RO+ cells in PBMCs of AD patients (25.61 ± 6.08%) was significantly higher than that of healthy controls (5.24 ± 1.15%) and Ps patients (3.72 ± 1.97%) by FACS analysis. The strong immunoreactivity for TARC was detected in epidermal KCs, vascular endothelial cells and dermal infiltrating cells in the lesional skin both in the acute and chronic phase of AD. Immunoreactivity for TARC was virtually negative in normal skin and weakly positive in the lesional epidermal KCs of Ps. Taken together, these results demonstrates that the serum TARC level is closely associated with disease activity of AD and that TARC may play an important role in the pathogenesis of AD.

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Altered Cytokine Production and Expression of Costimulatory Molecules in Monocytes and Monocyte-Derived Dendritic Cells from Atopic Dermatitis Patients

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Atopic dermatitis (AD) is associated with a bias of T helper cells to show increased IL-4 and reduced IFN-γ production. Antigen presenting cells (APC) such as monocytes (MO) and dendritic cells (DC) strongly influence Th1/Th2 deviation by changing their production of cytokines and their expression of costimulatory molecules. In this study, we examined whether atopic MO or DC differ from those of healthy controls in the production of cytokines and expression of costimulatory molecules. We studied 12 AD adult patients demonstrating more than 2000 U per ml of IgE. Controls were 12 age-matched nonatopic healthy adults. We examined the production of GM-CSF, IL-1β, IL-10, IL-12p40, and IL-12p70 and the expression of CD23, CD40, CD54, CD80, CD86, and HLA-DR by MO stimulated by SEA, streptococcal pyrogenic exotoxin A (SPEA), LPS, PHA, IL-4, IL-1/TNF-α, or IFN-γ. We also examined the production of IL-1β, IL-10, IL-12p40 and IL-12p70, and the expression of CD23, CD86, and HLA-DR by monocyte-derived DC stimulated by LPS, agonistic anti-CD40 antibody, or IFN-γ. Statistical analysis of the obtained data demonstrated (1) increased production of IL-10 and decreased production of GM-CSF by AD MO stimulated with LPS, and decreased IL-12p40 production by AD MO with various stimulations (2) increased CD23 up-regulation by AD MO stimulated with IL-4, increased CD54 up-regulation by AD MO stimulated with IL-1/TNF-α or SEA, and decreased CD86 up-regulation by AD MO stimulated with IL-1/TNF-α (3) decreased production of IL-12p40 and IL-12p70 by AD DC stimulated with LPS or anti-CD40 antibody, and (4) decreased CD86 up-regulation of AD DC stimulated with anti-CD40 antibody. These data suggest that APC from AD patients play a role in their Th2-deviated pathogenic phenotype expression of helper T cells.

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The Role of Neutralizing Antibody to Staphylococcal Superantigen in Patients with Severe Atopic Dermatitis

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Our previous studies demonstrated that T cells from the patients with severe atopic dermatitis (AD) were prone to undergo apoptosis following staphylococcal enterotoxin B (SEB) stimulation. In contrast to this *in vitro* phenomenon, we observed only few apoptotic T cells at the AD lesions with staphylococcal infection. To clarify this discrepancy, we hypothesized that antiapoptotic activity was present in the serum of the patient. Here we examined whether or not sera of AD patients had any influence on T-cell response to SEB. Sera from 27 severe AD patients (IgE5000 U per ml), 9 patients with psoriasis vulgaris and 15 healthy controls were used for this study. Normal peripheral blood mononuclear cells (PBMC) were cultured in the presence of serially diluted sera, followed by stimulation with SEB (0.1 μg per ml). [^{3}H]-thymidine incorporation was measured after three-day culture. In 13 of 27 AD patients, serum titers (expressed as the reciprocal of the highest serum dilution resulting in 50% suppression of PBMC proliferation) were more than 10, while sera from psoriasis patients and healthy controls demonstrated no suppression or very low titers. Depletion of IgG from AD sera by protein G sepharose abrogates the suppressive effect on PBMC proliferative response, whereas eluted IgG fraction had a suppressive activity. Western blotting analysis demonstrated the presence of anti-SEB IgG in sera of AD patients. To examine the role of anti-SEB IgG *in vivo*, NC/Nga mice (as model mice for AD) were treated with AD sera before intracutaneous injection with SEB producing strain of *S. aureus* (SEB-*S. aureus*) which cause severe dermatitis in these mice. AD sera, but not normal sera, prevented SEB-*S. aureus* inducing severe dermatitis. Our findings suggest that severe AD patients had high titers of anti-SEB neutralizing antibody that may down-regulate inflammatory responses induced by SEB in the patients.

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Production of Soluble Mediators by Inhalant Allergen-Stimulated Human Blood-Derived Dendritic Cells from Atopic vs. Nonatopic Individuals

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To elucidate pathophysiological functions of antigen-presenting cells (APC) in atopic diseases, the present study compared the capacity of human blood-derived dendritic cells (DC) from atopic and nonatopic individuals to produce immunologically key-mediators upon treatment with inhalant allergens. Treatment of differentiated, immature, 98% pure, T-cell-free preparations of DC with various concentrations of house dust mite or grass pollen extracts for up to 72 h dose-dependently induced DC maturation, release of MCP-1 and induction of IL-12p35 mRNA, but no release of IL-12 and IL-10. Full cytokine release was achieved by treatment of DC with either endotoxin (LPS) or a cytokine cocktail consisting of IL-1β, TNFα and IL-6. Remarkably, in 7/8 paired investigations of age and sex-matched individuals, maximum production of IL-10 and MCP-1 was 1.5–2-fold higher in atopic vs. nonatopic DC, whereas IL-12 production did not show significant differences. LPS- as well as cytokine cocktail-induced cytokine production was modulated by 48 h pretreatment of DC with inhalant allergens, resulting in dose-dependent super-production of IL-10 and MCP-1. In contrast, IL-12 production was inhibited by very high and very low concentrations of inhalant allergen pretreatment, resulting in a bell-shaped dose–response curve of IL-12 release. As a more physiologic model for DC activation in human skin, untreated as well as inhalant allergen-primed DC were challenged with supernatants from untreated or activated cultured normal human keratinocytes (HNK). DC responded upon supernatants from HNK with significant cytokine production (MCP-1, IL-10). This cytokine production could be increased, either if DC were pretreated with inhalant allergens for 48 h or if HNK were activated by e.g. UV-irradiation. Our data demonstrate differential regulation of inhalant allergen-induced DC cytokine production and they suggest geniune DC-dependent differences in the release of Th2-like cytokines in atopic vs. nonatopic individuals. In human skin, DC activation may be modulated by cells of the surrounding microenvironment, i.e. keratinocytes. Taken together, it may be speculated that the pathophysiological basis for atopic diseases is found in cells of the unspecific branch of the human immune system rather than in cell types determining specificity of immune responses.

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Phorbol Ester-Induced Apoptosis Associated with Syncytia Formation in Human Monocytic Cell Lines Established from Peripheral Blood of Patients with Atopic Dermatitis and Psoriasis

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12-O-tetradecanoylphorbol-13-acetate (TPA) known as a tumor promoter and protein kinase C activator, induces apoptosis which is mediated by tumor necrosis factor (TNF)-α as in human monocytic cell line U937cells. We studied the regulation of apoptosis by TPA in YAA and YAP cells established from peripheral blood of patients with atopic dermatitis and psoriasis, respectively. When YAA, YAP and U937 cells were treated with 1 ng of TPA for 24 h, the cell growth of each cell line was inhibitted and the viability of YAP, YAA, and U937 cells were 18%, 35%, and 90%, respectively. TPA-treated cells stained with 4′,6′-diamino-2-phenylindole dihydrochloride (DAPI) showed morphologically apoptotic change. However, the amount of TNF-α in the culture supernatants was not significantly different. Interestingly, TPA induced remarkable syncytia formation in YAA and YAP cells, but not in U937 cells. These syncytia were also associated with apoptotic cells as judged by the DAPI stainning. These results suggest that YAA and YAP cells seem to be usuful for investigation of giant cell formation as well as apoptosis in human monocytic cells. Our data also suggest that these cells might be usuful for basic researches in treatments of lymphoma and in granulomatous skin diseases.

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SB239063, a p38 Map Kinase Inhibitor, Inhibits Development of Psoriatic Lesions as Effectively as Cyclosporin A in C.B-17 SCID Mice Adoptively Transferred with Naïve T Cells from B10.D2 Splenocytes

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We have previously reported that adoptive transfer of naïve T cells isolated from B10.D2 donors into C.B-17 SCID (SCID) mice, produced symptoms of disease similar to psoriasis. Here we evaluated the ability of SB239063, a novel p38 MAP kinase inhibitor, to prevent onset or reduce symptoms of disease. SCID mice were injected IV with 0.33×10^6 naïve T cells from B10.D2 donors, and evaluated weekly for signs of disease for a duration of 8 weeks. Our results showed that SB239063 significantly inhibited the onset of psoriatic lesions by 62.5%, while Cyclosporin A and CTLA4-Ig had 66.7% and 100% reduction in development of lesions, respectively. Our compound also reduced the number of absolute number of granulocytes in blood by 72.8% as determined by CBC analysis, while Cyclosporin A and CTLA4-Ig reduced the number by 30.3% and 80.9%, respectively. Cytokine analysis of supernatants of skin homogenates revealed that SB239063 had a 54.9% inhibition of IL-1β, a 39.4% inhibition of IFN-γ, a 64.7% inhibition of TNF-α, and no effect on IL-4 levels. Cyclosporin A demonstrated a 61.2% inhibition of IL-1β, a 59.9% inhibition of IFN-γ, a 21.6% inhibition of TNF-α, and also had no effect on IL-4 levels. CTLA4-Ig demonstrated a 90.8% inhibition of IL-1β, an 88.4% inhibition of IFN-γ, a 96% inhibition of TNF-α, and also had very little effect on IL-4 levels. Taken together, these data show that SB239063 reduced symptoms of psoriasis as effectively as Cyclosporin A, however, neither compound was as effective as CTLA4-Ig.

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LFA3TIP Affects Epidermal CD8+ T Cells *In Vivo* and Down-Regulates Keratin 16 and Ki-67 Expression in the Psoriatic Plaque

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Six patients received 12 weekly doses of LFA3TIP, a fully human LFA-3/IgG1 fusion protein. 6 mm biopsies were performed at baseline and weeks 7 and 13 (1 week after final dose). Biopsies were snap frozen and analyzed for immunohistochemical markers (CD3, CD4, CD8, and CD2), Ki67, keratin 16, and epidermal thickness. Pan-T cell staining with CD3 demonstrated a decrease in the numbers of epidermal T cells present in 4/6 patients while dermal T cells decreased in 3/6. Staining for CD2 was similar to the CD3 data and results were also consistent with staining for CD4 and CD8. CD4 staining was primarily in the dermis and was only slightly decreased in 3/6 samples at week 13, while CD8 staining was primarily in the epidermis and showed significant decreases in 4/6 patients by week 7. Notably, the epidermal effects at the week 7 biopsy antedated the dermal changes. Dermal changes including reduction in Ki67 staining (5/6 samples) and a diminution of keratin 16 expression (4/6 patients) were of lesser magnitude and were evident between week 7 and 13. These data suggest a differential sensitivity between dermal CD4+ T cells and epidermal CD8+ T cells to LFA3TIP. By having a significantly greater effect on epidermal CD8+, most of which are likely to be CD45RO+, LFA3TIP may preferentially reduce the activated cells directly interacting with keratinocytes in psoriasis, while relatively sparing CD4+. Moreover, since changes in epidermal T cells preceded other immunohistochemical changes (e.g. Ki-67 and keratin 16), the data provide *in vivo* proof of the proximal nature of CD8+ T cells in the psoriatic lesion. Altering CD8+ T cells specifically with relative sparing of dermal CD4+ T cells appears to reduce the activity of psoriasis. Biologics (e.g. LFA3TIP) capable of targeting specific lymphocyte subsets can give important insights into the pathogenesis of T cell-mediated autoimmune disorders.

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Role of Transforming Growth Factor-β-1 in the Th1/Th2 Mechanisms of Vasogen's Immune Modulation Therapy: Potential Role in Psoriasis

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Transforming Growth Factor-β-1 (TGF-β1) is known to play a major role in the modulation of immune responses and the down-regulation of inflammation. Increased TGF-β1 production correlates with protection and/or recovery from inflammatory and autoimmune diseases. It is proposed that there are populations of regulatory T cells that exert their action primarily by secreting this cytokine. Recently, Vasogen's Immune Modulation Therapy (IMT) has been introduced in the treatment of a number of inflammatory and autoimmune disorders. IMT is believed to down-regulate T cell- mediated inflammatory responses by suppressing the Th1 arm of the immune system. IMT involves modifying autologous blood by controlled exposure to an oxidizing agent and UVC, at an elevated temperature *ex vivo* and then administered by intramuscular injection. The initial randomized, double blind clinical trial in patients with moderate to severe psoriasis has shown that 40% of patients receiving IMT experienced a clinically significant benefit (greater than 50% improvement in maximal PASI score), double that seen in the placebo group. The objective of this present study was to evaluate the role of TGF-β1 in the mechanisms of the immune modulating effect of IMT using contact hypersensitivity (CHS) as a model. Mice treated with IMT-processed blood demonstrated a significantly lower CHS response than controls. RT-PCR demonstrated that IMT treatment induced a significant up-regulation of TGF-β1. This was associated with a two-fold down-regulation of IFN-γ and IL-6. We have previously shown that IMT works through an IL-10 dependent mechanism. The results of these studies suggest that beneficial effects of IMT on psoriasis are mediated through modulation of cytokines.

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IFN-γ+ Epidermal T Cells and IL-8+ Neutrophils in Psoriasis

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IFN-γ and IL8 are believed to be important mediators in the cytokine cascade of psoriasis. It is not known (1) wether epidermal T cells produce IFN-γ within the lesion and (2) whether the elevated levels of IL8 in psoriatic scales are predominantly of keratinocyte origin or whether they are derived from other cells within psoriatic skin. We performed immunohistochemical studies to try to determine *in situ* localization of IFN-γ+ cells and IL-8+ cells in psoriatic lesions by using formalin-fixed, paraffin-embedded sections. Considerabe amounts of IFN-γ+ cells were detected in infiltrateds of the papillary dermis. IFN-γ+ cells were found to be present in the epidermis. The pattern of IFN-γ+ staining appeared to be a combination of intracellular staining in lymphoid cells and extracellular depostion in the surrounding areas. IL-8 was positively stained in the vast majority of neutrophils but not in the mononuclear cells, macrophages, or keratinocytes. IL-8+ neutrophils were seen both in Munro's microabcesses in cases of psorisis vulgaris and in a small spongiform pustule and much larger macropustules of Kogoj in cases of pustular psoriasis. Some IL-8-positive neutrophils were observed in the upper dermis of pusrular psoriasis. Our data suggest that (1) psoriatic epidermal T cells produce and secrete IFN-γ within the lesion and these T cells are involved in the pathogenesis of psoriasis, and that (2) neutrophils are one of the sources of IL-8 in psoriasis and the IL-8 autocrine and/or paracrine system functions in the formation of the microabcesses and pustules in psoriasis.

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Use of Expression Cloning to Identify Autoantigens in Psoriasis

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Psoriasis is a very common disease, affecting approximately 2% of the world's population. Severity ranges from a minor cosmetic concern to a severe skin disease with life-threatening flares and disabling arthritis. There is strong evidence that the disease is driven by a T cell mediated immune response in the skin. However, the antigen or antigens to which these T cells react have not yet been defined. In this project, we plan to identify these antigens using the SEREX method, which is an expression cloning method widely used in the study of tumor antigens. This method does not make any assumptions about the nature of the antigens and is significantly more rapid than other methods used to directly characterize T cell antigens. It is based on the observation that cell mediated immune responses can be associated with parallel humoral responses to the same antigens. Although the humoral component is not necessarily an effective component of the immune response, the presence of these antibodies has proven useful in the SEREX method by allowing immunoscreening of a cDNA library with autologous serum to identify antigens which are also recognized by T cells. The first aim of this project is to create cDNA expression libraries from psoriatic epidermis, screen these with autologous serum, sequence cDNAs encoding antigens, and analyze the sequences for possible functions, homologies, and mutations which may be relevant in the pathogenesis of psoriasis. We have created four of these libraries and have started work screening. At this point, none of the autoantigens identified has an obvious clear relationship to the pathogenesis of psoriasis, such as homology to a streptococcal protein. The next aim is therefore to assess which antigens most commonly elicit a humoral immune response in psoriasis patients compared with normal controls using a dot blot assay. We conclude that this work demonstrates the feasibility of applying the SEREX method to the study of psoriasis. This technique should also be applicable in the study of other T cell mediated skin diseases such as lichen planus, vitiligo, and alopecia areata. With continued progress, we expect this approach will significantly improve understanding of the pathogenesis of psoriasis and may provide new targets for treatment in the future.

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Analyzing of Lesional T-Cells in Psoriasis by Flowcytometry

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The cellular infiltrate in psoriasis has been characterised extensively by immunohistology and revealed a T-cell predominance. The aim of the present study was to further characterise these cells by multiparameter analyses. We determined phenotypic markers and performed short-term functional assays with T-cells using a novel protocol for mechanic disaggregation of skin specimens. Skin biopsies and venous blood samples were obtained from 10 patients with currently untreated psoriasis. The cellular composition of the lesional lymphocytic infiltrate showed a T-cell predominance. The higher frequency of CD4+ than CD8+ cutaneous T-cells resulted in a high CD4+/CD8+ T-cell ratio. Our immunophenotypic characterisation revealed a high proportion of activated, proliferating (HLA-DR+, Transferrinreceptor (CD71)+) memory/effector (CD45RA–, CD45RO+) CD4+ and CD8+ T-cells in psoriatic plaques. The higher frequency of such cells compared to that in peripheral blood indicates their selective cutaneous accumulation. The majority of lesional T-cells expressed the adhesion molecule CD11a and the chemokine receptor CXCR3. Finally, we determined the intracellular cytokine formation in cutaneous T-cells from 4 patients after short-term stimulation. A high number of CD4+ and in particular CD8+ T-lymphocytes expressed IFN-γ and TNF-α, whereas IL-2 and IL-4 producing T-cells were only rarely detected. Consequently, the IFN-γ/IL-4 ratio was high, indicating a type 1 cytokine predominance. This is of particular interest since activated T-cells and type 1 cytokines are considered to be key players in psoriasis. Our investigations demonstrated a activated, memory/effector type 1 immunophenotype in psoriasis and indicated that the mechanic disaggregation followed by flow cytometric analysis is a fast and useful approach for multiparameter characterisation of skin-infiltrating lymphocytes.

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Induction of Psoriasiform Histology by NK Cells is a General Phenomenon of both Psoriatics, and Non-Psoriatics

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This study was performed to ask whether psoriasis is a unique pathological response of epidermis of psoriatic patients, or whether NK cells can induce psoriatic changes in skin from nonpsoriatic donors. Human, and animal protocols were approved by the appropriate institutional review committees. Human nonlesional skin from 3 psoriatics (NP), as well as from 7 nonpsoriatics (NN) was grafted onto SCID mice (CB-17 Prkdc-scid). Lymphocyte lines with NK activity were generated by culture of PBMC in IL-2100 U per ml for 14 days. Cells were phenotyped, and tested for INF-γ production, and NK activity by CTX against K562 cells, then injected into the human skin grafts. The following permutations were tested: (1) autologous psoriatic NK cells in NP skin, (2) allogeneic normal NK cells in NP skin, (3) autologous normal NK cells in NN, skin (4) allogeneic normal NK cells in NN skin, (5) allogeneic psoriatic NK cells in NN skin. After 6 weeks, the grafts were harvested and analyzed for histology, immunohistochemistry, epidermal thickness, proliferation (Ki-67), and K16. Injection of autologous psoriatic NK cells into NP skin resulted in classic psoriasis histology with significant ($p < 0.05$) increase in epidermal thickness, and proliferation. NK cells also induced expression of epidermal HLA-DR, ICAM-1, CD1d, and K-16. Injection of normal NK cells into autologous NN skin, and allogeneic NK cells into NN or NP skin, induced psoriasiform changes, with less elongation of the rete ridges. The ability of NK cells to induce psoriasiform changes is a general phenomenon. NK cells from both autologous, and allogeneic donors were able to induce psoriasiform changes in normal skin from both psoriatics and non-psoriatics. Superantigen stimulation was not necessary. Psoriasiform changes are part of the normal response repertoire of normal skin from non-psoriatics.

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Interleukin-17 Production by Isolated Circulating Skin-Homing T Cells from Psoriasis Patients

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The T cell–keratinocyte interaction plays a relevant role in the pathogenesis of psoriasis. IL-17 is a proinflammatory cytokine produced by T cells that is involved in keratinocyte activation. The subset of circulating memory T cells with cutaneous tropism (CLA) positive cells in psoriasis contains increased percentage of activation markers like HLA-DR, CD25 and CD69. This is an indication that circulating memory CLA positive cells may traffic between blood and psoriasis lesions and may be implicated in the pathological mechanisms. The purpose of this study was to determine the production of IL-17 by purified CLA positive and CLA negative cells memory T cells from individuals with psoriasis (n = 4), atopic dermatitis (n = 3) and healthy controls (n = 3). Cell isolation of both CLA positive and negative memory T subsets was performed by an immunomagnetic procedure and cells were activated with anti-CD3 and anti-CD28 after 5 days in culture. IL-17 was analyzed by ELISA in the 48 h supernatant after activation. Our data indicate that the CLA positive subset produce higher amount of IL-17 than the CLA negative subset. Among the three groups studied, IL-17 production by CLA positive cells was: psoriasis (2.6 ± 0.3 ng per ml), controls (1.0 ± 0.4 ng per ml) and atopic dermatitis (0.3 ± 0.2 ng per ml). The levels of IL-17 produced by the CLA negative subsets were: 0.7 ± 0.1; 0.3 ± 0.07; and 0.07 ± 0.06 (ng per ml) for the three groups, respectively. This data indicate that IL-17 is produced in high amounts by circulating skin-homing T cells during psoriasis. This is the first data showing IL-17 production by circulating CLA positive memory T cells.

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Decreased IL-10 Production by Psoriatic Peripheral Blood Mononuclear Cells Stimulated with Streptococcal Superantigen

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The strong association of acute guttate psoriasis and streptococcal throat infection has suggested a role of streptococcal antigens in the pathogenesis of psoriasis. We have reported that psoriatic peripheral blood mononuclear cells (PBMCs) showed significantly lower responses to cytoplasmic membrane-associated protein (CAP) isolated from group A β-hemolytic streptococci, a kind of streptococcal superantigen. To evaluate the abnormal cytokine production by psoriatic PBMCs to streptococcal superantigen, CAP, we compared the production of 4 different cytokines, i.e. IL-4, IL-5, IL-10, and IFN-γ, by PBMCs between 13 psoriatic patients and age-matched 16 healthy controls after the stimulation with CAP or two staphylococcal superantigens, staphylococcal enterotoxin A (SEA) or E (SEE). When PBMCs were stimulated with CAP, the production of IL-10 was significantly lower by psoriatic PBMCs than by those from healthy controls, whereas that of IL-4, IL-5, or IFN-γ was not different between the two groups. Such a significant decrease in IL-10 production by psoriatic PBMCs was not observed when they were stimulated with staphylococcal superantigens. Flow cytometric analysis of intracytoplasmic IL-10 demonstrated that the defective IL-10 production by psoriatic PBMCs was recognized in both CD3+ T cells and CD14+ monocytes. There was a significant positive correlation between IFN-γ production by PBMCs and the proliferation of Vβ8+ T cells which was preferentially stimulated by CAP. These data demonstrating the defective IL-10 production by psoriatic PBMCs stimulated with streptococcal superantigen can explain the reason why only psoriatic patients evolve the sustained and Th-1 deviated skin lesions after streptococcal upper respiratory infection.

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Interleukin 4-Induced Immune Deviation as Therapy for Psoriasis

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The inflammation leading to the manifestation of psoriasis is dominated by interferon-γ producing type 1 T cells. Immune deviation of type 1 into interleukin 4 (IL-4) dominated type 2 responses improves inflammatory autoimmune diseases in mice. Since IL-4 is most potent in inducing a type 2 phenotype in T-cells of human origin, we investigated safety and efficiency of systemic IL-4 therapy in patients with severe psoriasis (phase Ib study). Five groups, each including 4 patients, received increasing doses of IL-4 over 6 weeks. The first group received the lowest dose. IL-4 dose was increased by factor of 2 in each group. The follow up included weekly PASI documentation, skin biopsy and analysis of immune parameters potentially affected by IL-4. A total of 22 patients was recruited, 20 terminated the study. Two interrupted and one had side-effects grade II. In 18/20 patients PASI decreased by 60–80% within six weeks and no rebound occured during the 6 weeks of follow up. Patients treated with higher doses of IL-4 had statistically better improvement than patients treated with low doses of IL-4. Efficient therapy was associated with a decrease of skin infiltrating cells and a normalisation of the epidermal structure. Blood cell counts remained unchanged during IL-4 therapy except of an increase of the eosinophil fraction. Cytokine analysis of freshly isolated lymphocytes revealed an increase in $IL\text{-}4^{+}CD4^{+}$ T-cells, whereas the fraction of $IL\text{-}2^{+}CD4^{+}$ T-cells remained unchanged. Monocytes showed a higher CD23 expression during IL-4 therapy. Importantly serum IgE levels and IFN-γ production to unrelated recall antigens were not influenced. Thus, the data strongly suggest that IL-4 therapy improved psoriasis by immune deviation affecting exclusively the recently activated T-cells.

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Therapeutic CD11a Ligation Transmodulates Surface Expression of β1 and β2 Integrins on T-Lymphocytes

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Anti-CD11a is a nonlymphocyte depleting, humanized monoclonal antibody under investigation for treatment of moderate to severe plaque psoriasis. CD11a (aL integrin) combines with CD18 (β2 integrin) to form LFA-1, the ligand for ICAM. β2 integrin also forms heterodimers with aM (CD11b) or ax (CD11c) to constitute the related adhesion molecules, Mac-1 and p150,95. Lymphocytes also bear two integrins from a separate family which bind to VCAM, a4β1 (CD49d/CD29, VLA-4) and a4β7. Using FACS analysis, we have measured CD11a, CD11b, CD11c, CD18, CD49d, CD29, and β7 on circulating T-lymphocytes from psoriatic patients before and after 14 days of treatment with 2.0 mg/kg/week SC anti-CD11a. At baseline, LFA-1 was expressed on 99%, VLA-4 on 88%, CD11b on 31% and CD11c on 4% of circulating T-cells. At Day 14 of treatment, surface expression of CD11a was down-regulated 86% (p = 0.002), as reflected by binding with a noncompetitive antibody. Assay with a competitive antibody showed 95% of residual surface CD11a was occupied by the therapeutic monoclonal. CD18 surface expression was reduced by a mean of 86% (p = 0.005). The frequency of CD11b+ cells was reduced by a mean of 65%, but the MFI values for the remaining positive cells was unchanged. Neither the proportion or MFI staining changed for CD11c + cells. Surface expression of VLA-4 was reduced by a mean of 72% (range 68–77%, p = 0.0001), with consonant reductions in β1 or β7 subunits. Down-regulation of surface integrins was not observed on lymphocytes after short-term incubation *in vitro*. These data suggest a novel *in vivo* effect of CD11a ligation leading to transmodulation of related and unrelated integrins. Since endothelial cells in psoriatic plaques up-regulate both ICAM and VCAM, combined down-regulation of LFA-1 and VLA-4 may effectively inhibit cutaneous T-cell trafficking, leading to the reduction in plaque T-lymphocyte number observed in patients treated with anti-CD11a.

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Alefacept Inhibits IFNγ Production by Normal and Psoriatic Peripheral Blood T cells and Enhances the Action of UVB

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Psoriasis is mediated, in part, by activated T-cell production of IFNγ. Alefacept (human LFA-3/IgG1 fusion protein, LFA3TIP, currently being developed under trade name AMEVIVE(tm)) shows inhibitory effects on T cells *in vitro* and *in vivo*. Phase 3 clinical trials of alefacept are ongoing in psoriasis. UVB irradiation remains one of the most effective treatments of psoriasis, and we previously reported that a single *in vivo* UVB exposure can selectively decrease T-cell IFNγ production. To investigate effects of alefacept on T-cell IFNγ production, PBMC from normal individuals (n = 7) or psoriatic patients (n = 7) were activated and IFNγ production was measured by flow cytometry. For 8 μg per ml alefacept-treated nonpsoriatic PBMC, the number of IFNγ+ T cells decreased in 5/7 cases (20–90% reduction), increased in 1/7 or remained unchanged in 1/7. In psoriatic PBMC, 8 μg per ml of alefacept caused a decrease in IFNγ production in 6/7 patients tested, with a mean 56 ± 0.12% reduction (p < 0.005). PBMC populations could be divided into 2 groups based on IFNγ production, high (10%) or low (< 10%) IFNγ+CD3+. When considered separately, both nonpsoriatic and psoriatic high producers were effectively inhibited by 8 μg per ml alefacept, with a mean reduction of 56% and 65%, respectively. By contrast, low producers showed little inhibition. AntiFcγ RI & III mAb pretreatment abolished the reduction of IFNγ by alefacept. When PBMC populations were pretreated with UVB irradiation (0–20 mJ per cm^2), alefacept enhanced UVB-induced apoptosis and further decreased IFNγ by 32.2% (p = 0.009, n = 3). These results indicate that alefacept inhibits T-cell IFNγ production, that an interaction with FcγR bearing cells is required, and that its combination with UVB may prove effective in reducing the number and activity of Th1-type cells in the psoriatic lesion.

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A Three-Month Multiple-Dose Toxicity Study of Alefacept in Baboons

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The toxicity of alefacept (human LFA-3/IgG1 fusion protein, LFA3TIP, currently being developed under the trade name AMEVIVE(tm)) was evaluated after repeat IV administration to baboons. 24 baboons were randomized into 4 groups of 3 males and 3 females. Control animals received saline; treated animals received alefacept doses of 0.1, 2, or 40 mg per kg per week (equal to, 20 times, 500 times the intended clinical dose [7.5 mg IV], respectively) for 13 weeks. On Day 93, 1 male and 1 female from each group was sacrificed. The remaining animals were evaluated for 7 months and sacrificed on Day 316. No mortalities or clinical signs of toxicity were observed during the dosing or postdose recovery phases. In addition, no evidence of infectious compromise or neoplastic changes were observed. Alefacept-related changes were limited to dose-dependent decreases in peripheral and tissue-associated lymphocyte counts. Despite use of doses 500 times the intended clinical dose, no T-cell subset was fully depleted. The effects on lymphocytes showed evidence of saturability. Peripheral CD2+ and CD4+ T-cell reductions plateaued at approximately 80% below baseline in the 2- and 40-mg per kg per week groups. No gross abnormalities were noted in any animal. At the tissue level, alefacept resulted in distinct decreases in cellularity of T cell-specific regions of the spleen and lymph nodes. By Day 316, peripheral lymphocyte counts had returned to predose baseline levels in the 0.1-mg per kg per week group and continued to recover toward baseline levels in the 2- and 40-mg per kg per week groups. Tissue changes remained evident in the spleens of the recovery sacrifice animals from the 40-mg per kg per week group; however, the extent of these changes was less evident than in those animals evaluated on Day 93. In conclusion, despite prolonged lymphocyte reductions, no signs of toxicity or infectious compromise were observed following weekly administration of alefacept at doses in excess of 500-fold greater than those administered clinically. Effects on peripheral lymphocytes were saturable. Doses consistent with the intended clinical dose showed complete lymphocyte recovery.

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Autoantibodies to 155 kDa and Se Antigens in Patients with Clinically Amyopathic Dermatomyositis

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Patients (pt) with confirmed clinically-amyopathic dermatomyositis (C-ADM) have not been studied systematically for myositis-related auto-antibodies (aab). In this study, an ANA-positive serum from a pt (Se) with C-ADM immunoprecipitated (IPP) a series of protein bands from HeLa cell extract of approx. 155, 140, 120, 95, 90, and 50 kd. By IPP, the 155 and 140 kd bands matched in size those of anti-155 kd, an aab that has also been found in some pt with classical DM. Absorption studies indicated that this represented anti-155 kd in association with a second aab labeled anti-Se (the 90-95 kd doublet). Sera of 17 additional C-ADM pt were studied (systematic testing for myopathy by EMG, biopsy and imaging was not performed on all pt, i.e., the designation ''clinically-amyopathic''). 3 of the 17 were members of the same family. By IPP, sera from 12 of 17 (71%) showed a protein of 155 kd consistent in size and appearance with anti-155 kd; 3 of the 5 others showed a protein of roughly similar size but somewhat different in appearance. 5 also showed the typical doublet of Se (29%), while 6 others showed one band possibly consistent with Se. No associated nucleic acid was consistently seen in the immunprecipitates (IPPs). Western blot of anti-Se IPPs showed that 5 of 18 (28%) (including Se) reacted with bands in the 95 kd region. The 3 family members also reacted with at least one band in this region. 10 of 18 (56%) reacted with one or more additional bands (110 and 120 kd) IPP by Se. 6 of 18 sera (33%) reacted by blot with the 155 kd band, all of which also reacted with other bands of Se IPPs. Combining blot and IPP data, 16 of 18 (89%) C-ADM pt showed aab to a 155 kd protein and/or an Se protein. None of the 18 pt had a myositis-specific aab (e.g., Jo-1). Thus, anti-155kd and anti-Se aab are common in C-ADM, while MSAs are not.

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Analysis of the Effector Cells of Autoimmunity in Transgenic Mice Overexpressing CD40L in the Epidermis

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CD40/CD40L interactions play a pivotal role in immune-mediated inflammatory responses via activation of antigen presenting cells (APC). To investigate the effects of continuous activation of the APC of the skin, the Langerhans cells (LC), CD40L expression was targeted to the epidermis of mice using the keratin-14 promoter. As previously reported, 80% of the CD40L transgenic (Tg) mice developed dermatitis on the ears, face, tail and/or paws. Surprisingly, they also develop a systemic autoimmune disease as evidenced by the presence of ANAs, antidsDNA antibodies and the typical internal organ involvement. Compared to their littermates, Tg mice had a 90% decrease in LC numbers within the epidermis yet increased numbers within the dermis. DC in the hyperplastic LN draining from inflammed skin of Tg mice exhibited enhanced levels of the activation markers I-A, CD80 and CD86. Increased numbers of FITC+/CD11c+ in the LN of Tg mice compared to WT mice following topical application of the hapten FITC were found indicative of enhanced emmigration of CD40–activated LC. The infiltrate of the skin consisted of CD4+ and CD8+ T cells and BM8+ cells. To further substantiate the role of T cells as effector cells of local autoimmune skin inflammation, T cells from the draining LN of Tg mice were transferred into nontransgenic recipient mice. Only the transfer of T cells from Tg mice evoked the development of skin lesions similar to those found in the Tg mice. Dermatitis was not dependent on B cells or immunoglobulins as CD40L Tg backcrossed into the B cell deficient strain JHT also developed dermatitis. These findings indicate that *in situ* activation of LC by CD40L in the skin not only leads to chronic inflammatory dermatitis but also to systemic mixed connective tissue-like autoimmune disorders. CD40L Tg have the potential of being an excellent model for dissecting the T cell and immunoglobulin dependent pathways in the pathogenesis of these type diseases.

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Induction of Autoimmune Skin Disease by Passively Transferred T cell?@Receptor Transgenic T Cells into Keratin 14-Ovalbumin Transgenic Mouse

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To understand the mechanisms involved in immunological tolerance to skin-associated antigens, we have developed transgenic (Tg) mice that express a model self-antigen, membrane bound chicken ovalbumin (OVA), under the control of a keratin 14 (K14) promoter. These K14/OVA Tg mice express OVA mRNA in the epidermis, do not exhibit any phenotypic abnormalities, and have similar CD4/CD8 distributions as their littermate controls. Proliferative responses of draining lymph node (LN) cells were assessed after subcutaneous immunization with OVA + CFA and were markedly reduced in the Tg mice compared with their littermate controls, suggesting that the Tg mice were tolerant to OVA. When CD8+ T cells from OVA specific T cell receptor ($V\alpha2/V\beta5$) Tg mice (OT-1) were injected IV into the K14/OVA Tg mice on day 0, OT-1 T cells expanded and accumulated in LN draining the skin (0.5%, 6.4%, 10.5% of cells in LN on day 2, 4, 7, respectively). The accumulated T cells that were identified by their $V\alpha2/V\beta5$ transgene exhibited activation markers (CD25+, CD44Hi, CD62LLo). Furthermore, GVHD-like skin lesions (accumulation of dermal mononuclear cells and the presence of dyskeratotic cells in the epidermis) appeared by day 7. There was also significant reduction in thymic size in the Tg mice that received the OT-1 CD8+ T cells. These studies demonstrate that the K14/OVA Tg mouse can serve as a good model for the investigation of the pathogenesis, treatment and potential prevention of cell-mediated autoimmune reactions in skin.

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Topical Application of Interleukin-7 Plasmid DNA Reduces Severity of Skn(Skin)-Directed Autoimmunity

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Previous work has shown that transfer of autoreactive lymphocytes targeted against the mouse epidermal cell antigens, Skn, can cause skin lesions in recipient animals. This response is contingent not only on the introduction of autoreactive cells, but also, on the inability of recipient animals to control the attack. Control of the autoimmune response can be restored by cotransfer of normal CD4+ T cells and is evident by reduced severity of lesions. Analysis of cytokine gene expression at the skin site of lesion control revealed a significant increase in interleukin (IL)-7 mRNA. The current study examined whether exogenous IL-7 delivery by topical application of DNA could control lesions without the cotransfer of normal CD4+ T cells. Animals were prepared for Skn-directed autoimmune attack and 5 micrograms of plasmid DNA containing the IL-7 gene was applied daily to skin sites adjacent to the location of potential lesions. Control animals received either plasmid DNA without the IL-7 gene or no plasmid. The animals were observed for 7–12 days and lesions were scored on a scale of 1–5. Delivery of plasmid containing IL-7 significantly reduced the severity of lesions (lesion score 1.4), as determined by both gross and histologic examination, when compared to either control group (lesion score 3.3), $p < 0.01$. Lesion control was attributed to the presence of plasmid IL-7 gene expression and not to endogenous production of IL-7, in that, mRNA for plasmid IL-7 identified by a FLAG marker accounted for 99% of the messages detected at the skin site. These results strongly suggest that topically applied cytokine DNA can be delivered to target skin sites and may be of value in the treatment of certain skin disorders.

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Phenobarbital-Induced Eruption: The Drug Preponderantly Stimulates Th2 Cells Bearing Particular TCRBVs without its Processing

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Phenobarbital has a high potential to elicit adverse reactions compared to other drugs. Although phenobarbital hypersensitivity is thought to be mediated mostly by T cells specific to the drug, its precise mechanism remains unfully elucidated. To characterize T cells reactive with phenobarbital, we generated drug-specific T cell line cells (TCC) from peripheral blood mononuclear cells from patients with phenobarbital-induced eruption. We found that the majority of TCC were Th2 cells and some of clones expressed cutaneous lymphocyte-associated antigen. CD4+TCRBV13.1+ phenobarbital-specific clones were generated from three individuals with different HLA haplotypes, and these cells had an ability to proliferate in response to the antigen with processing-impaired, fixed antigen-presenting cells (APC). The lack of necessity of processing and the common usage of particular TCRBV in different individuals suggest that the response is clearly different from the conventional one with regard to T cell–APC interaction. Our observations are important to reveal the unsettled mechanism of immune response against such small compounds as drugs.

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Exocytosis of Cells Stained by Anti-CYP2C9 Antibody in the Mefenamic Acid-Induced Fixed Drug Eruption

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Although skin tests with offending drugs have been tried for the diagnosis of drug eruption (DE), there is no information about the nature of antigen. Metabolic processes of drugs would be preceded to become antigenic, and cytochrome P450s (CYPs) may participate an antigen formation through the generated metabolite. The aim of this study was to examine the role of CYPs in fixed drug eruption (FDE). 3 cases of FDE caused by mefenamic acid were included in the study. 2 samples were obtained from a biopsy of lesion, and one was from a biopsy of positive patch test. The paraffin-embedded specimens were stained with anti-CYP isozyme antibodies, 1A1, 2C9, 2E1 and 3A4. To identify the cells stained with the antibodies, antikeratin antibody and antibody for leukocyte common antigen (LCA) were also used. Specimens from negative patch test and lesions caused by doxyxycline were used for control to compare the results. All sites induced by mefenamic acid showed exocytosis of inflammatory cells stained with LCA. The cells were mostly stained by anti-CYP2C9 antibody, which was not observed in control specimens. The results supported that CYPs play a role in occurrence including antigen formation of FDE, and inflammatory cells including lymphocytes would be an important source of the CYPs, although the reason for recurrence of FDE on the same sites still remains to be clarified.

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Peptide ELISA for Detection of Circulating Autoantibodies Against Desmoplakin in Erythema Multiforme

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Autoantibodies directed against desmoplakin I and II have been identified recently in a subset of patients with erythema multiforme. Subsequently, a specific peptide sequence (YSYSYS) localized within the very carboxy terminal domain of desmoplakin thought to be responsible for the assembly of keratin filaments to the desmosomal plaques was identified as antibody binding epitope. Sera were tested by immunoblotting, immunoprecipitation and dot blot analysis to varify the presence of peptide specific antidesmoplakin autoantibodies. These methods are laborious and time consuming. The aim of this study was to establish an enzyme-linked immunosorbent assay (ELISA) to evaluate serum samples from patients with erythema multiforme for the presence of peptide specific antidesmoplakin autoantibodies. A total of 7 serum samples of two patients with defined peptide specific autoantibodies, serum samples of 20 additional patients with erythema multiforme with and without antidesmoplakin autoantibodies characterized by immunoblotting and immunoprecipitation and sera of 20 healthy volunteers, were processed for ELISA on microtiter plates coated with the corresponding synthetic peptide. Correlation of ELISA, immunoprecipitation and dot blot was 100% and so was the correlation of the results from ELISA and immunoprecipitation. We thus introduce a peptide ELISA as a practical tool for large scale screening for peptide specific antidesmoplakin autoantibodies.

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An *In Vitro* Organ Culture Model for Toxic Epidermal Necrolysis

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Purpose: To determine in vitro effect of serum obtained from patients with acute toxic epidermal necrolysis (TEN) on normal human conjunctiva (NHC), normal human skin (NHS) and normal human buccal mucosa (NHBM). Methods: Serum samples were collected from patients in the acute phase of TEN and stored at −80°C. The NHS, NHC and NHBM were cut into 2-3 mm^2 pieces and cultured separately with 10-30% (v/v) of serum from TEN patients at 37°C for 1-12 hrs, 24, 36, 48 and 60 hours. Normal human serum was used as control. Each tissue was then embedded in a paraffin block. The blocks were cut and sections were stained with hematoxylin and eosin, and stained with immunoperoxidase using antibodies to human Fas and Fas Ligand (Fas-FasL). Results: Acantholysis and vacuolar degeneration were dominant histopathologic features observed in the epithelium of NHS, NHC, and NHBM cultured in 30% TEN serum after 24 hrs of incubation. These findings were not observed in the epithelium of NHS, NHC and NHBM cultured with 30% normal human serum. In addition, by immunoperoxidase staining, compared to normal serum treated tissues, an increased expression of Fas and FasL was seen in the epithelium of TEN serum-treated tissues. Conclusions: The TEN serum induced identical morphologic and immunohistochemical changes in vitro in the epithelium of NHS, NHC, and NHBM. This may represent an in vitro model for TEN. The Fas/FasL mediated apoptosis may play a role in the pathogenesis of TEN.

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Factors Secreted by B16 Melanoma Cells Induce Nitric Oxide Production in RAW 264.7 Macrophages – is Nitric Oxide Contributing to B16 Melanoma Mediated Immunosuppression and Tumor Facilitation?

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Previous work in our lab has shown that B16 mouse melanoma cells secrete yet unidentified tumor facilitating factor(s) (TFF). TFF promotes systemic tumor development in both nude and C57BL/6 J mice. TFF also systemically immobilizes macrophages away from sites of inflammation, inhibiting both delayed type hypersensitivity (DTH) and lymphocyte mitogenic responses. Prompted by recent evidence suggesting that Nitric Oxide (NO) plays a major role in the suppression of antitumor defenses, DTH responses, and lymphocyte stimulation by antigens, we decided to examine the effect of TFF on NO production by RAW 264.7 mouse macrophages. *In vitro* treatment with TFF induced RAW 264.7 macrophage spreading which was similar to that observed with mouse macrophages treated *in vivo* with TFF and plated on glass cover- slips. TFF also resulted in a marked, dose dependent up-regulation of NO in RAW 264.7 cells, but not in B16 melanoma cells. The NO up-regulation was not detected at 3 h after TFF treatment, but was detected at 15 h. Flow cytometry of RAW 264.7 cells grown with or without TFF revealed inducible Nitric Oxide Synthase (iNOS) up-regulation after treatment for 16 h with TFF. The RAW 264.7 macrophages treated for 24 h with TFF and washed extensively every 24 h were still producing NO after 72 h, and returned to basal NO production after 96 h. By using preparative isoelectric focusing we have determined that the proteins at pI 4.1 and 4.8 coincide with crucial measurements of TFF activity such as maximal NO production, macrophage spreading, and tumor facilitation. The fraction at pI 4.1 promoted tumor growth in 6 of 10 mice, and that at pI 4.8 in 5 of 5 mice. In the control mice only 1 of 5 mice grew tumors. These findings raise the possibility that NO up-regulation induced by TFF may be involved in systemic immunosuppression and tumor facilitation.

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α(1,3) Fucosyltransferase VII Deficient Mice Exhibit Impaired Cutaneous Tumor Immunity

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Fucosyltransferase VII is required for the generation of the glycosylation epitope on PSGL-1 that defines CLA, an E-selectin ligand important in the homing of memory T cells to skin. Mice deficient in this enzyme have impaired cutaneous hypersensitivity reactions, as well as other leukocyte defects. To test the ability of these mice to mount effective antitumor immunity, wild type and FucTVII−/− C57BL/6 (B6) mice were injected intradermally with live J558L BALB/c plasmacytoma cells. As expected, tumors grew vigorously in syngeneic BALB/c mice, while they were promptly rejected without any evidence for growth in wild type B6 mice. In FucTVII−/− mice the tumors grew progressively until day 11, reaching an average size of 1.62 cm. Over the next three weeks, they began to diminish in size. On day 6 after tumor implantation, the site was analyzed by histology. WT B6 mice showed a significant local inflammatory response consistent with tumor cell rejection, while both B6 FucTVII−/− and BALB/c mice showed tumor growth without an observable host inflammatory response. To determine whether this defect in cutaneous tumor immunity was due to a lymphocyte defect, FucTVII deficient mice were injected intravenously with WT C57BL/6 lymph node cells at the time of tumor challenge. The transfer of WT T cells from skin draining lymph nodes led to a dramatic decrease in the time required for tumor rejection in FucTVII deficient hosts; in contrast, WT T cells derived from mesenteric nodes did not confer this activity. To determine whether the defect in tumor immunity was skin specific, J558L cells were injected intraperitoneally. In BALB/c mice, they grew progressively as predicted. In both WT B6 and FucTVII−/− B6 mice, no intraperitoneal tumor growth was perceptible. This is the first demonstration that FucTVII deficient mice have a skin-specific impairment of tumor immunity, and suggests that organ-specific homing T cells may play an important role in immunosurveillance and rejection of tumors by the immune system.

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Melanoma CNS Metastases Display a Different Chemokine Secretion Pattern than Skin or Lymph Node Metastases

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Migration and distribution of melanoma cells during metastasis is poorly understood. Recently, chemokines, an increasing family of potent chemoattractants, were shown to play an important role for the distribution and migration of leucocytes during inflammation. In the present study we investigated chemokine and chemokine receptor profiles in melanoma metastases from different tissue sites (n = 20; 8 skin (S), 6 lymph node (LN) and 6 CNS (CNS) metastases). Assessment was performed by PCR based semiquantitative mRNA expression analysis and immunohistochemical analysis of biopsy samples as well as cell lines. Predominant expression of SDF-1α and MIP-3α was found in melanoma samples during a PCR based screening process. In skin metastases 6 out of 8 and 4 out of 8 samples expressed MIP-3α and SDF-1α, respectively. In lymph node metastases 4 out of 6 and 5 out of 6 samples expressed MIP-3α and SDF-1α, respectively. In contrast, only 1 out of 6 CNS metastases expressed MIP-3α and 2 out of 6 SDF-1α. Expression pattern of MIP-3α and SDF-1α was confirmed by immunohistochemistry. Next we investigated the expression of MIP-3α and SDF-1α receptors CCR6 and CXCR4. Both receptors were found to be expressed on melanoma cells *in vivo* and *in vitro*. Our data suggest that chemokine expression pattern in CNS metastases differ from pattern seen in skin and LN metastases. This finding might have implications for tissue homing and redistribution of melanoma cells.

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Serum S100 Level is Predictive of Recurrence-Free Survival in Patients with Intermediate Risk Melanoma

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S100 has been recently explored as a serum marker for malignant melanoma; however, its utility to predict prognosis remains controversial. In this study we examined whether the presence of S100 in the circulation is predictive of the prognosis of melanoma in patients with different stages of disease. We measured serum levels of S100 in 126 patients with melanoma (58 with AJCC stage IIB/IIIA, 48 with stage IIIB, and 20 with stage IV disease) using a double sandwich ELISA. We found that 31% of patients with stage IIB/IIIA, 42% of those with stage IIIB and 40% with stage IV melanoma were positive for S100. Recurrence-free survival was significantly shorter in stage IIB, IIIA, and IIIB patients who were S100 positive compared to S100 negative patients, i.e. by Kaplan Meier analysis the mean time recurrence-free survival was 11.9 ± 0.6 months vs. 20.3 ± 0.8 months ($p = 0.009$). There appeared to be a correlation between the stage of disease and the prognostic significance of S100 level, as correlation with prognosis was best in patients with stage IIB, IIIA, and IIIB disease and absent in patients with stage IV disease. These results suggest that serum S100 levels may be a prognostic marker in patients with resected melanoma at intermediate risk of recurrence.

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IFN-γ Reduces Melanoma Tumor Antigen Expression and Recognition by Cytotoxic T Cells

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Boosting the host immune response is currently the treatment of choice for metastatic melanoma. Most CTL are reactive against melanosomal differentiation antigens, such as MART-1. Achieving complete remissions by innovative immunotherapeutic strategies utilizing peptides derived from target antigens is frequently hampered by metastatic lesions that evade immune recognition. In this study, we sought to define an immune escape pathway by exploring the influence of a T cell derived cytokine, IFN-γ, present within the microenvironment of melanoma tumor cells. Examination of primary and metastatic melanoma lesions by qRT-PCR revealed up to 188 fold more abundant IFN-γ transcripts when compared to control skin. Using laser capture microdissection and immunohistology, the majority of IFN-γ producing T cells were localized to tumor stroma, accompanied by a halo of reduced MART-1 expression by the adjacent tumor cells. IFN-γ (100–1000 U per ml) enhanced HLA-DR expression, but markedly suppressed expression of melanosomal antigens (e.g. MART-1, gp100 and TRP-1) in M14 melanoma cells as shown by immunohistology and flow cytometry, reducing MART-1 levels by 65%. IFN-γ mediated suppression of MART-1 was regulated at the transcriptional level as determined by Northern blot analysis, and was dependent on the continued presence of IFN-γ. To determine whether exposure of tumor cells to IFN-γ modulates recognition by CTL, HLA-A201 melanoma cells treated with or without IFN-γ were combined with A42 MART-1 reactive T cells. Cytotoxicity was reduced up to 78% in a 51Cr release assay at effector: target ratios of 10:1 by IFN-γ pretreatment, and could be restored by addition of synthetic MART-1 peptide. Recognition of normal melanocytes by A42 CTL was not reduced by IFN-γ. Thus, while IFN-γ is a type-1 cytokine generally regarded as enhancing cellular immune responses, these novel findings indicate that IFN-γ can also reduce target antigen expression and recognition of melanoma cells by CTL. The apparent sustained recognition of CTL to IFN-γ exposed melanocytes in contrast to melanoma cells may explain how some patients develop extensive vitiligo despite metastatic tumor sites escaping immune recognition.

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Identification of Antibodies in the Serum of Patients with Melanoma-Associated Retinopathy

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Melanoma-associated retinopathy (MAR) is a paraneoplastic syndrome manifesting in association with cutaneous melanoma. Signs and symptoms include night blindness, light sensations; (photopsias), visual loss, and reduced b-waves in the electroretinogram. Retinal degeneration is believed to result from the production of antibodies reactive with melanoma-associated antigens which cross-react with epitopes of retinal bipolar cells. We recently analysed 26 melanoma patients for the presence of subclinical MAR symptoms using scotopic electroretinography, static and kinetic perimetry, nyctometry and the Farnsworth panel test for color vision. 6 patients showed all typical signs of MAR, 17 were suspicious of MAR and only 3 had no definite signs of MAR. We conclude, that some patients with cutaneous melanoma may experience a subclinical retinal involvement suggesting MAR with an incidence more common than previously suspected. In pursuing the antigenic source of the sensitization process we found that melanomas from the primary and distant metastases of two of the MAR patients contain cells that express antigen(s) that react with rabbit antiretina serum. The focus of antibody activity was clearly localized upon the "rosette" cell population within the malignancy. These data provide further evidence that the MAR syndrome may be linked to the expression of antigen(s) able to induce the production of autoantibodies cross-reactive with retina tissue, and that the "rosette" cell population may be the prime contributor to the induction of this immunologic anomaly. Our findings lead us to hypothesize that under circumstances yet to be defined, loss of tolerance to the "rosette"-expressed antigen(s) occurs and if this aberrant reaction is accompanied by leaks in the blood–retina barrier, the MAR syndrome develops.

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MART-1/MelanA and gp100/PMel17 Immunodominance Revisited: Direct Enumeration of Frequency of Melanoma Differentiation Antigen (MDA)-Specific T-Cells in Tumor Metastases TIL Cultures

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Melanoma differentiation antigens (MDA) such as MART-1/MelanA and gp100/PMel17 are considered immune dominant based on the observation that they are often identified as targets of tumor infiltrating lymphocytes (TIL) originated from HLA-A*0201 expressing patients with melanoma. Furthermore, clinical relevance was attributed to this dominance based on the impression that the adoptive transfer of gp100-recognizing TIL was associated with clinical responses in a small group of patients. Here, we enumerated MDA-specific T-cell precursor frequency (Tc-pf) in TIL using tetrameric HLA/epitope complexes (tHLA) and functionally characterized their responsiveness to cognate epitope by cytokine release assay. Tc-pf were enumerated in 11 fresh tumor preparations and 17 TIL adoptively transferred into HLA-A*0201 bearing patients. Furthermore, the kinetics of TIL expansion *in vitro* was followed in four lesions by serial documentation of Tc-pf. MART-1 or gp100-specific T cells could be detected in 5 and 2 of the 11 fresh preparations and in 5 and 2 of the 17 adoptively transferred TIL. With one exception, Tc-pf in fresh material and TIL ranged between 0.5 and 2.1%. Tc-pf was not significantly higher in TIL whose administration was associated with clinical response. These data provide evidence that MDA immune dominance and their clinical in the context of HLA-A*0201 has been probably overrated.

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Expression of Melanocortin-1 Receptor (MC-1R) in Normal, Malformed, and Neoplastic Skin Glands and Hair Follicles

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Proopiomelanocortin-derived peptides with neuroimmunomodulating functions have been identified in many nonpituitary tissues including the skin. To exert their function they bind to melanocortin (MC) receptors. Recently, expression of melanocortin-1 receptors could be detected in normal skin particularly in hair follicle epithelia, sebocytes, and sweat gland epithelia. In the present study, the expression of melanocortin-1 receptor (MC-1R) immunoreactivity in various cutaneous malformations and neoplasms with adnexal differentiation was examined. A specific antimelanocortin-1 receptor-antibody was applied to a total of 95 skin samples of hamartomas, cysts, hyperplasias and benign or malignant neoplasms of eccrine, apocrine, sebaceous glands, and hair follicles. Immunoreactivity was widely preserved in all of the samples as compared to normal skin and only slight variations with regard to maturation of the neoplasm were found. The adnexal epithelia showed a preferential intracytoplasmic granular in addition to an intercellular staining pattern. Immunoelectron microscopical investigations revealed expression of MC-1R both along the cell surface and within tubular endosomes suggesting internalisation of the receptor. In summary, MC-1 receptor expression is widely preserved in various adnexal malformations and neoplasms of the skin. Since alpha-melanocyte stimulating hormone has been shown to increase sebum production in rodents, our findings suggest a potential role of the MC-1R also in sweat gland function, follicular proliferation, as well as in tumorgenesis.

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Expression of Melan-A/MART-1 Antigen as a Prognostic Factor in Primary Cutaneous Melanoma

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In the past decade, analysis of the immunogenic properties of human melanomas has allowed the identification of a wide array of melanoma associated antigens recognized by specific cytolytic T lymphocytes. This study assessed the expression of one of these, the Melan-A/MART-1 antigen, by immunohistochemistry using mAb A103 in 73 primary cutaneous melanomas and its correlation with tumor staging and patient survival over 10 years. Melan-A/MART-1 was expressed in 90% of primary tumors, with loss of expression increasing with Breslow thickness ($p = 0.003$). Kaplan-Meier analysis demonstrated a significantly reduced disease-free interval ($p = 0.002$) and overall survival rate ($p = 0.005$) for patients not expressing this antigen. The effect of Melan-A/MART-1 absence on survival was maintained when compared to Breslow thickness in a Cox multivariate analysis ($p = 0.05$). The poor prognosis of such patients increased when considering those presenting with a primary melanoma and a Breslow thickness of ≥ 1 mm (disease-free interval $p < 0.0001$; overall survival rate $p = 0.0006$). Our study suggests that assessment of Melan-A/MART-1 expression, besides being useful in the diagnosis of primary cutaneous melanoma and the monitoring of patients in Melan-A/MART-1 vaccination trials, may also provide an additional factor to refine the prognosis of patients with a primary melanoma thicker than 1 mm as measured by Breslow.

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Cutaneous T Cell Lymphoma is an Antigen-Driven Malignancy Stimulated by Dendritic Cells

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Cutaneous T cell lymphoma (CTCL) cells have been difficult to propagate limiting study of the factors which stimulate their uncontrolled growth. To overcome these problems, we have developed a long-term culture system in which CTCL lymphocytes and dendritic antigen-presenting cells (APC) proliferate for at least 3 months. The cultures contain aggregates of CTCL cells around dendritic cells (DC) reminiscent of Pautrier microabscesses. Proliferation requires contact of the CTCL cell T cell receptor (TCR) with the cocultured transitional DC. Leukapheresed CTCL cells were cultured at 30×10^6 per well in media containing GMCSF/IL4 and IL2/IL7. Proliferation was measured with 3[H]-thymidine. Cells were immunophenotyped for membrane CD83, class II, CD80, CD86 and cytoplasmic CD83 and DC-Lamp. In replicate cultures, reproducibly established from 5 CTCL patients, both the CTCL cells (15x inc) and the DC proliferated (4x inc) throughout the 3 month culture period. Either cell type cultured alone with supportive cytokines died within 3 weeks. CTCL cells were CD4+, CD8–Vβ+ and retained the initial clonal PCRγ and β-chain rearrangements. The DC were a mixture of CD14–, class II+, CD86+, CD80+ immature cells (60%) and CD83+, class II+, CD80+, DC-Lamp+ (40%) maturing DC. The proliferation of the CTCL cells was inhibited with antibodies (AB) to the TCR Vβ and DC growth was inhibited by CD40 AB. Neutralizing AB to CTCL cell IL10 and the addition of apoptotic tumor cells resulted in a 37% increase in DC maturation to CD83+/class II+ cells. These studies present the first method for readily establishing long-term cultures of CTCL cells and maturing DC. This culture system represents an *in vitro* model for study of the *in vivo* pathognomonic hallmark of CTCL,the Pautrier microabcess. Moreover, these studies are the first demonstration of an antigen-driven T cell malignancy and the reproducible establishment of APC cultures that can mature into DC *in vitro*.

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

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

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Functional Characterization of a $CD8^+CD56^+CD45RA^+$ Malignant T-Cell Line Derived from a Patient with Unusual $CD8^+$ Sezary Syndrome

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We established a malignant T-cell line from blisters developing on erythrodermic skin of a patient with unusual $CD8^+$ Sezary syndrome. The cells showed a $CD3^{low}$ $CD2^{high}$ $CD4^-$ $CD7^-$ $CD8^+$ $CD45RA^+$ $CD45RO^-$ $CD56^+$ CLA^+ phenotype. More than 40% of the patient's peripheral blood lymphocytes (PBL) and 98% or more of cells from blisters had this phenotype. Southern blot analysis with probes for TCR Cβ1 of the patient's PBL and skin specimen exhibited identical rearranged nongerm line bands. Fresh and long-term-cultured tumor cells proliferated well in response to IL-2, IL-7 and Con A when cultured for 3 days. Both IL-2 and Con A induced tumor cell apoptosis and necrosis. On the other hand, IL-7, known as a Sezary cell growth factor, prevented the cells from apoptosis by retaining a high level of Bcl-2 expression and allowed them to live over 2 months, while IL-2 induced a marginal enhancement of Bcl-2 expression only for the first 2 days of culture. By RT-PCR for mRNA expression of cytokines, the cells transcribed the message for IL-10 and IFN-γ but not for IL-2, IL-4 or IL-5 even in the presence of Con A. As assessed by ELISA of culture supernatants, they produced IL-10, IFN-γ, TNF-α and TGF-β1 upon stimulation with Con A. Although the amount of TGF-β1 produced by IL-7-stimulated cells is higher than that by Con A-cultured cells, IL-7 did not stimulate them to secrete IFN-γ or TNF-α, demonstrating selective production of TGF-β1 by IL-7. The cells did not substantially express TGF-β receptor type II (TGF-βRII) and, unlike normal T cells, its expression was not enhanced by Con A stimulation. These results demonstrated that this unusual IL-7-dependent $CD8^+$ Sezary cells expressed a surface NK cell marker and had a NK1-type cytokine profile (IL-10 and IFN-γ) despite their virtual T-cell origin. The production of TGF-β1 and the loss of TGF-βRII expression suggest that this unique malignant T cell is capable of escaping from tumor immunosurveillance by virtue of TGF-β1 without self-suppression by this cytokine, leading to aberrant growth of tumor cells and disease progression.

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Impaired CD40 Ligand Expression and Signaling in Sézary Syndrome Results in Defective Interleukin-12 and TH1 Cytokine Production: Restoration by Recombinant Soluble CD40 Ligand Stimulation

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Depressed cellular immunity in Sézary syndrome is considered to be partly due to defective TH1 cytokine production, but its mechanism remains to be defined. CD40-CD40 ligand (CD40L) interactions are known to contribute to the development of TH1 responses by inducing the production of interleukin-12 (IL-12). Here we show by FACS analysis that CD4+ T lymphocytes of Sézary patients (7/7 cases) have a marked defect in CD40L expression upon activation *in vitro*. This defect is not restricted to the CD4+/CD7- population, and is more pronounced in patients with a high circulating tumor burden. In these patients, production of IFN gamma, TNF alpha and IL-12 by peripheral blood mononuclear cells (PBMC) after *in vitro* T cell activation was also strongly reduced, and most markedly in those with a high tumor burden. As a similar defect was reproduced in healthy control PBMC by CD40L blockade, and no defect in CD40 expression was detected in Sézary patient's APC, defective CD40L expression and signaling was thought to be a possible cause. Indeed, incubation of PBMC from Sézary patients with recombinant soluble CD40L + IFNγ, but not IFNγ alone, partially restored TNFα and IL-12 production. These data suggest that defective CD40L expression contributes to reduced TH1 cytokine production in Sézary syndrome, and that this can be restored *in vitro* by recombinant CD40L, representing another potential immunotherapeutic approach to treatment of Sézary syndrome.

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Control of Growth and Malignant Progression of Oncogene SV40-Driven Tumors by CD4+ T Cells

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RIP1-Tag2 transgenic mice express the oncogene SV 40 T antigen (Tag) under the control of the rat insulin promotor in β cells of the pancreas, early during embryogenesis. They develop tumors at 6 weeks of age and become first anergic and T-ag specific CD4+ T cells are then deleted. At about 12 weeks, these tumors grow invasively and cause premature death due to a profound hypoglycemia around week 14. Earlier data show that interferon-γ-producing CD4+ Th1 cells are highly effective in controlling transplanted tumors. Therefore we investigated the effect of Tag-specific Th1 cells on growth and the development of MHC class II-negative endogenous tumors, driven by a strong promotor. CD4+ T cells were derived from transgenic mice, with a T cell receptor specific for the Tag peptide (362–384) and stimulated *in vitro* with peptide and anti-IL-4 mAb to generate Tag-specific Th1 cells. These Th1 cells were transferred at weekly intervals, starting at 7 weeks after initiation of hyperplasia and neoangiogenesis. Blood glucose was documented biweekly and animals were sacrificed when blood glucose was < 30 mg per dL. Tumor-growth, tumor-specific immune response and angiogenesis were monitored every 3 weeks. Adoptive transfer of Th1 cells prevented the decrease of blood glucose for almost 3 month, surprisingly without inducing diabetes. Death was delayed from 14 to 25 weeks. The therapy was associated with the appearance of tumorspecific Th1 cells in draining lymph nodes and a strong infiltrate of CD4+ and CD8+ T cells in the tumors. In contrast to experiments obtained with CD8+ T cells, Th1-therapy did not eliminate single adenomas but obviously delayed tumor growth and dedifferentiation, which is characterized by neoangiogenesis, development of dysplastic cells and invasive growth. The data suggest a hereto unexpected role for antitumor immune responses as they demonstrate not only that MHC class II-restricted Th1 cells are capable of breaking tolerance and controlling growth of MHC class II-negative carcinomas, but act predominantly by delaying the dedifferentiation-program dictated by the SV40 oncogene.

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Regulation of Transglutaminase 2 by Transforming Growth Factor-β1 in Dermal Fibroblasts

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Transglutaminase 2 (TGase 2) is a calcium-dependent enzyme which mediates covalent cross-linking between the?-carboxamide group of glutamine and the e-amino group of lysine. The enzyme is known to cross-link intracellular proteins and extracellular matrix proteins. Transforming growth factor-β1 (TGF-β1) has been reported to induce TGase 2 in hepatoma cells, ocular trabecular meshwork and epidermal keratinocytes. This study was performed to evaluate the role of TGF-β1 on TGase 2 expression and activity in dermal fibroblasts to explore its possible function in regulation of extracellular matrix formation. Dermal fibroblasts treated with TGF-β1 (0.2–5.0 ng per ml) were cultured in a 5% CO2 incubator for various times. Western blot analyses for TGase 2 and fibronectin and total TGase activity assay were performed with cell extracts, and RT-PCR for TGase mRNA expression was performed with total RNA extracts. TGase 2 expression was induced by TGF-β1 as time- and dose-dependent manners. In Western blot analysis, maximal expression of TGase 2 was observed 18 h after TGF-β1 treatment at a concentration of 1 ng per ml. Total TGase activities were concordant with the pattern of TGase 2 protein expression. TGase 2 mRNA expression was induced earlier than that of protein expression, indicating the transcriptional regulation of TGase 2 by TGF-β1. TGF-β1 also induced fibronectin expression in dermal fibroblasts by Western blot analysis. The present study shows that TGase 2 is induced by TGF-β1 in dermal fibroblasts, which in turn cross-links extracellular matrix proteins, including fibronectin. Our results suggest that TGF-β1 has an important role in the dermis to control formation of extracellular matrix expression by mediation of TGase 2.

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Epidermal Growth Factor Up-Regulates Transforming Growth Factor-β Receptor Type II in Human Dermal Fibroblasts via p38 Mitogen-Activated Protein Kinase Pathway

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TGF-β receptors (TβRs) are serine/threonine kinase receptors that bind to TGF-β and propagate intracellular signaling through Smad proteins. TβRs are known to be repressed in some human cancers and expressed at high levels in several fibrogenetic diseases. In the present study, we demonstrated that EGF up-regulated the expression of typeII TGF-β receptor (TβRII) mRNA and proteins, but not type I TGF-β receptor (TβRI), in human dermal fibroblasts. Actinomycin D, an RNA synthesis inhibitor, significantly blocked the EGF-mediated up-regulation of TβRII mRNA expression, whereas cycloheximide, a protein synthesis inhibitor, did not block this up-regulation. In addition, EGF treatment did not significantly affect the TβRII half-life. EGF-mediated induction of TβRII expression was inhibited by the treatment of fibroblasts with a specific p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580, whereas mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059 did not block up-regulation of TβRII. Additionally, EGF induced TβRII promoter activity, which was significantly blocked by SB203580, not by PD98059. Co-transfection of the TβRII promoter with dominant negative forms of p38 MAPK significantly abolished the level of EGF induction. These results indicate that EGF-mediated induction of TβRII expression occurs at the transcriptional levels, does not require de novo protein synthesis, and involves the p38 MAPK signaling pathway. EGF induction of TβRII expression may participate in a synergistic interplay between EGF and TGF-β signaling pathway.

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Expression of p53, p21^{WAF-1} and Bax Proteins, and Apoptosis in Ischemia-Reperfusion of Rat Skin

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We investigated p53, p21^{WAF-1} and Bax proteins, and apoptosis to elucidate the cellular response to ischemia-reperfusion of skin. Rat left lower limb was amputated retaining bone and femoral vessels, and vessels were clamped to produce ischemic condition. After 6 h, the clamps were removed, and the plantar skin was resected at various times up to 72 h after reperfusion. Expression of three proteins was detected by Western blot analysis. Apoptotic cell was detected using TUNEL assay. After reperfusion, the levels of p53 and p21^{WAF-1} proteins were significantly higher in the ischemia-reperfusion rats as compared with the sham-operated rats. However, the levels of Bax protein did not show a significant increase at any period studied. Apoptotic cells in both the epidermis and dermis was not evident as compared with the sham skin, which were similar to that in nontreated, normal skin. These results demonstrated that p53 and p21^{WAF-1} proteins were accumulated after 6 h ischemia of the skin during reperfusion. Moreover, it is suggested that accumulation of these proteins play an important role in survival of the skin, which may be caused by inducing growth arrest of the cells, not apoptosis.

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Human Papillomavirus Oncoprotein Expression Alters Cell Cycle Kinase Expression and Predisposes Animals to Cancer Development

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Human papillomaviruse (HPV) is a DNA tumor virus that has been implicated in the genesis of cancer in various squamous epithelia, including the epidermis. The HPV oncoproteins E6 and E7 are thought to promote cell immortalization and subsequent cancer development by inactivating the tumor suppressor proteins, p53 and pRB. To study the role of HPV in disease causation in skin and other surface epithelia, we have developed a transgenic animal model in which the viral E6/E7 oncoprotein-encoding reading frame is targeted to skin using the human involucrin promoter. These animals display extensive epidermal, oral and cervical hyperplasia, but no tumor formation. In the present study, we characterize the cell cycle regulatory protein status in the epidermis of these mice. We show that the hyperproliferation is correlated with increased expression of p53, p21, p27, cdk2, cdk4, cdk6, cyclin D1 and cyclin E. This phenotype suggests an important role for E7 in regulating cell cycle kinase function. In addition, treatment of the epidermis with 7,12-dimethylbenz(a)anthracene (DMBA) results in preferential papilloma formation in E7 mice vs. controls. Moreover, treatment of the oral cavity with 4-nitroquinoline-1-oxide (4NQO) results in the formation of large oral tumors in E6/E7 but not control mice. Our results suggest that the changes in cell cycle protein expression are a compensatory response to the presence of the E6/E7 oncoprotein, and the E6/E7-positive animals are predisposed to cancer formation when challenged with carcinogens.

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Inducible Expression of TGF-β1 in Papillomas Causes Rapid Metastasis

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TGF-β is believed to be a tumor suppressor at early stages, however, it has also been postulated to promote tumor invasion. To determine at which stage and by what mechanisms this functional switch occurs, we have generated gene-switch-TGF-β1 mice in which TGF-β1 transgene expression can be induced in skin tumors at specific stages. These mice were exposed to a chemical carcinogenesis protocol, which allows tumorigenesis to develop in progressive stages from benign papillomas to malignant carcinomas. TGF-β1 induction in early stage papillomas suppressed tumor formation and growth. However, at later stages, TGF-β1 transgenic papillomas exhibited a resistance to regression and an increase in malignant conversion. More strikingly, TGF-β1 transgene induction rapidly induced metastasis from tumors with a benign papilloma histotype. The metastasis-prone papillomas exhibited down-regulation of TGF-β receptors and their signal transducer, the Smads, and loss of the invasion suppressor E-cadherin/catenin complex. These molecules were lost only in malignant carcinomas in control mice. Furthermore, metastasis-prone transgenic papillomas exhibited elevated matrix metalloproteinases, and an increase in angiogensis which correlated with changes in expression of angiogenesis regulators. Our study provides *in vivo* evidence at both the cellular and molecular levels that TGF-β1 overexpression directly induces tumor metastasis by initiating events necessary for invasion. Down-regulation of TGF-β signaling components in tumor epithelia selectively abolishes growth inhibition, thus, switching the role of TGF-β1 from a tumor suppressor to a metastasis promoter. Given that TGF-β1 overexpression is frequently detected in malignant human tumors, screening expression levels of TGF-β1 and its signaling components may have an important impact on prognosis and therapeutic approaches.

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Smad3/AP–1 Interactions Control Transcriptional Responses to TGF-β in a Promoter-Specific Manner

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Smad proteins transduce signals from TGF-β receptors and regulate transcription of target genes either directly or in combination with other sequence-specific transcription factors. AP-1 sites and their cognate transcription factors also play important roles in the gene regulatory activities of TGF-β. In this report, we have investigated the functional interactions of the Smad and AP-1 transcription factors. We demonstrate that Smad and AP-1 complexes specifically bind to their cognate *cis*-elements and do not interact with each other on-DNA, whereas off–DNA interactions occur between Smad3 and both c-Jun and JunB. Using both artificial constructs specific for either the Smad or AP-1 signaling pathways or natural promoters known to be TGF-β-responsive, we have determined that Jun family members down-regulate Smad3-mediated gene transactivation whereas AP-1-dependent promoters are synergistically activated by Smad3 and Jun proteins. We propose a model where the presence of Smad- and/or AP-1-specific *cis*-elements within TGF-β-responsive genes allows dynamic modulation of gene expression, in contrast to the existing model where interactions between Smad and AP-1 proteins are merely an on/off mechanism to regulate TGF-β/Smad targets.

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Withdrawn

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Rho Family GTPases, Rac and CDc42, Play an Important Role in Regulation of Human Keratinocyte Migration on Collagen

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Rho family GTPases, including Rho, Rac and Cdc42, belong to the p21-RasGTPase superfamily and play critical roles in the control of cell mitogenesis and morphogenesis. Activation of Rho, Rac and Cdc42 causes re-assembly of the actin cytoskeleton, leading to the formation of stress fibers, lamellipodia and filipodia, respectively. The roles of the Rho family GTPases in the control of fibroblast migration, macrophage chemotaxis and neuronal cell pathfinding have been reported. Whether or not they also mediate the signal transduction in human keratinocytes (HK), leading to cell migration, has not been studied. We used two independent, well-established cell motility assays – the *in vitro* wound scratch assay and the colloidal gold track assay – to investigate the roles of the Rho GTPases. In this report, we show that blockade of Rho GTPase activation by Toxin B completely inhibited HK migration on collagen. We found that continued activation of the Rho family GTPases was required for the entire (16–20 h) period of the migration assay, because Toxin B was able to stop the migration at any time point. To study which specific Rho GTPase(s) was involved, we transfected HK with either constitutively active or dominant-negative forms of RhoA, Rac1 and Cdc42Hs, whose expression was under control of a CMV promoter in pRK5 mammalian expression vector. Overexpression of the constitutively active RhoA, Rac1 and Cdc42Hs had no significant effect on HK migration on collagen. However, overexpression of the dominant negative Rac1 and Cdc42hs, but not RhoA, markedly decreased the keratinocyte migration. One of the downstream effectors for Rac and Cdc42 is the p38-MAPK and our previous studies have shown that p38-MAPK plays an important role in HK migration on collagen (Li *et al* submitted). Taken together, this study suggests that the Rac/Cdc42/p38-MAPK pathway may mediate collagen signaling in migratory keratinocytes

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Human Serum but Not Plasma Promotes Migration of Human Skin Cells

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The inward migration of human fibroblasts into the wound bed (fibroplasia) and the migration of human keratinocyte across the wound bed (re-epithelialization) are critical events to heal skin wounds. The mechanisms of cell motility are not well understood, but are thought to be an orchestration of both extracellular matrix components and soluble growth factors. During an acute wound, serum is generated and therefore for the first time skin cells experience serum rather than plasma. In this study, we asked if serum and plasma were the same or different in terms of supporting fibroblast and keratinocyte migration. We used two independent, well-established cell motility assays – the *in vitro* wound scratch assay and the colloidal gold track assay. In both assays, cell motility was strongly supported by human serum (Migration Index/MI 30%) but not by plasma (MI < 5%). High Performance Liquid Chromatography (HPLC) analysis of human serum demonstrated that the motility activity was distributed in three major fractions of the total 70 fractions. Each of the three fractions alone, however, only supported cell migration no more than 30% of the maximal motility in complete whole serum. These data strongly suggest that there are multiple serum factors that contribute to human keratinocyte and fibroblast migration. Further, these pro-motility soluble factors are absent in plasma suggesting that soluble factors from platelets similar to PDGF are likely involved. Finally, these factors have dual *in vivo* wound repair functions capable of supporting both keratinocyte reepithelialization and the ingress of fibroblasts into the wound bed to repair the dermal defect.

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PKC Involvement in the Autoregulation of Glucose Transport in Skin Keratinocytes. Implications to Chronic Diabetes Skin Complication

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Glucose represents a major fuel for most mammalian cells, and a wide range of factors regulate its utilization. However, abnormally high levels of glucose, as occurs in diabetes, lead to the development of chronic complications. One of the tissues mainly affected by the diabetes state is skin. Nonetheless, the pathophysiology by which glucose affects skin function is not known. We have previously identified effects of glucose on skin keratinocytes. Incubating cells in high glucose led to decreased proliferation, altered differentiation and down-regulation of glucose transport and glucose metabolism. In the present work we have extended these studies to investigate the involvement of the Protein kinase C family of serine-threonine kinases (PKCs) in the autoregulation of glucose transport in skin keratinocytes. We have found that activation of PKCs mimicked the high glucose effects on glucose uptake, induced by glucose. Exposure of the cells to PMA, a known PKC activator, resulted in a decrease in 2dGlc uptake that was similar in magnitude and time course to that induced by high glucose. Moreover, incubating the PMA treated cells with 20 mM of glucose could not further down-regulate the glucose transport rate. Further corroboration of these results was found by incubating proliferating skin keratinocytes in high glucose and following activation of specific PKC isoforms. Incubating the cells in high glucose was associated with translocation and activation of specific PKC isoforms, mainly PKCs δ and ζ. These results were confirmed in cells overexpressing these isoforms. In addition, over expression of PKCδ and ζ resulted in changes in the glucose uptake rate into keratinocytes. Overall, these results suggest the involvement of PKCs in glucose mediated toxic effects on skin keratinocytes.

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Development of a Transgenic Model for Conditional Regulation of AP-1 Transcription Factors in Epidermis

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Past studies using genetic alterations in members of AP-1 transcription factors have demonstrated a critical role of these proteins in skin carcinogenesis. To address general contributions of AP-1 to skin development and specific stages of carcinogenesis, a dominant-negative Fos protein (A-Fos) was generated which abolishes AP-1 activity. A-Fos was placed under regulation of the tetracycline transactivator promoter (tet-A-Fos). These mice were crossed to mice expressing the tet-transactivator under keratin 5 promoter regulation (bK5-tTA), such that A-Fos is expressed in the absence of tetracycline (or the analog doxycycline, Dox) only in keratinocytes. When bK5-tTA mice were crossed with tet-A-Fos mice in the absence of Dox, a lethal phenotype resulted, in that no bi-genic mice were born (n = 6 litters). However, when breeders were kept on Dox through pregnancy, a normal Mendelian distribution of the genotypes was seen, as assessed by PCR. Expression of the tet-A-fos transgene was confirmed to basal cells as detected by immuno-staining for HA antigen (engineered in the A-fos transgene). Expression was confirmed by RT-PCR for A-fos in RNA from skin. Bi-genic mice, when removed from Dox at birth, develop normally, have normal skin, and are fertile. When exposed to the hyperplasiogenic and inflammatory agent phorbol 12-myristate 13-acetate (TPA), the A-fos bi-genics exhibited marked increases in inflammatory infiltration at 24 and 72 h relative to controls. When primary keratinocytes were isolated from newborns and grown without Dox, cell density decreased by 1 week and cell morphology changed from cuboidal to a large, flat, and senescent phenotype. Bi-genic cells in the presence of Dox, or single transgenic controls maintained normal cubodial morphology and growth patterns through 3 weeks in culture. In cultured cells there was no genotypic difference in expression of keratins 1, 5, and 10 suggesting the phenotype observed is not through a strict change in the developmental program. When the bi-genic cells were transformed with a Ha-Ras retrovirus, the cells died after 72 h while control cells evolved a morphology typically seen in transformed keratinocytes. Results indicate that AP-1 is required for prenatal development and influences both skin inflammation and carcinogenesis. However, they may not be essential for postnatal epidermal function under conditions of normal homeostasis.

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Expression of Placenta Growth Factor, Cyclooxygenase, and Thymus and Activation-Regulated Chemokine in the EpiDerm *In Vitro* Human Skin Equivalent

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In vitro skin equivalent models are finding increased utility as tools in basic skin research, safety assessment and product development processes. To further utilization for these purposes, we are currently defining a growing list of physiologically significant molecular endpoints to monitor the effects of experimental treatments on EpiDerm(tm) *in vitro* human skin tissue. In the present work, we describe the expression in EpiDerm of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), placenta growth factor (PlGF), and thymus and activation-regulated chemokine (TARC) at the protein and/or mRNA level. Irradiation of EpiDerm with UVB caused elevated levels of cyclooxygenase product prostaglandin E2 (PGE2) secretion into the culture medium. Experiments with selective COX inhibitors indicate a prominent role for COX-2 in UVB-induced PGE2 production. Reverse transcription polymerase chain reaction (RT-PCR) was used to detect both COX-1 and COX-2 message in EpiDerm. COX-2 message was induced upon exposure to UVB. Message for the angiogenesis-inducing factor PlGF was also induced by UVB-irradiation. Finally, a combination of interferon-γ and tumor necrosis factor-α induced the expression of the TH2 recruitment CC chemokine TARC. These endpoints expand the utility of *in vitro* human skin equivalents for studies involving skin inflammation, skin cancer, topical sensitization and related skin phenomena.

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Novel Glutamate Receptor-Mediated Signaling Found in Bioengineered and Human Skin

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Recent studies have shown the presence of predominantly located-neuronal receptors in cutaneous tissue. Glutamate receptors, which are the primary mediators of excitatory neurotransmission in the central nervous system, have been found in animal skin and cultured human keratinocytes, and their altered expression has been observed in an animal model of wound healing. However, the expression of glutamate receptors has not been analyzed in human skin and/or models of human wounding. Our hypothesis is that these receptors may play an important role in human cutaneous calcium signaling. For these experiments, we used both normal human skin and a bilayered skin construct (BSC), comprised of human keratinocytes and fibroblasts, which is able to reepithelialize itself after injury. The expression pattern of a glutamate receptor was examined with immunohistochemistry using a monoclonal antibody to subunit 1 of the N-methyl-D-aspartate receptor (NMDAR1). Normal human skin showed a strong NMDAR1 staining pattern in the stratum malpighii, eccrine structures, sebaceous glands, and selected fibroblasts. The epidermal staining pattern was granular and peripheral, and the greatest staining intensity was found in basal cells. Uninjured BSC epidermis showed a similar pattern of NMDAR1 expression to that found in human epidermis. However, 24 h after injury by meshing, BSC showed markedly increased NMDAR1 expression in a polar pattern in migrating keratinocytes. These studies provide the first direct evidence for the presence of glutamate receptor-mediated signaling in human skin *in vivo*, and suggest that altered expression or function of NMDARs may play a role in pathological states where calcium-dependent mechanisms are prominent. Our work also points to glutamate receptor-mediated signaling as being important in wound healing and the process of reepithelialization.

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Induction of Multiple Types of Skin Tumors in Mice Overexpressing an Activated Gli2 Mutant

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The Sonic hedgehog (Shh) signaling pathway, operating through the Gli family of transcription factors, plays important roles throughout embryogenesis and in cancer development. Knock-out and transgenic mouse studies implicate Gli2 as the key transcriptional effector mediating Shh signaling in skin both under normal conditions and when pathologically activated in basal cell carcinomas (BCCs). Molecular analysis of Gli2 functional domains reveals a potent repressor in the amino-terminus: its removal in the Gli2ΔN2 mutant increases transcriptional activity up to 10-fold in cultured keratinocytes. Skin-targeted overexpression of this mutant using a K5 promoter in transgenic mice (*K5-Gli2ΔN2*) results in development of a variety of tumor types including trichoblastomas, cylindromas, basaloid follicular hamartomas, and very rarely, BCCs. This is in striking contrast to results obtained using mice overexpressing full-length Gli2, which exclusively produce BCCs. Tumors developed in multiple founders with the highest expression of *Gli2ΔN2* mRNA and protein, while those with lower transgene expression levels were either normal or exhibited hair follicle dysplasia. Northern blot analysis revealed a positive correlation between *Gli2?N2* mRNA levels and the degree of Shh pathway activation, based on expression of the Shh target genes *Gli1* and *Ptch1*. Our data point to a crucial role for the amino terminus of Gli2 in specifying BCC development in transgenic mice.

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IL-1α Overexpression Accelerates Head and Neck SCC in Mice

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The role of chronic inflammation in the development of head and neck squamous cell carcinoma (SCC) remains controversial. To address this question, transgenic mice that overexpress the pro-inflammatory cytokine IL-1a in basal epithelium (called TgIL-1.2) were studied for their susceptibility to oral cancer. Overexpression of IL-1ain the oral cavity was confirmed by immunohistochemistry and ELISA analysis of gingival IL-1α levels. TgIL-1.2 mice and wild type littermate controls were subjected to the carcinogen 4-Nitroquinoline-1-oxide (4NQO) by thrice weekly topical applications to the palate. Two dosing periods, 12 and 17 weeks, were evaluated. Strikingly, 100% of TgIL-1.2 mice (n = 14) developed SCC in at least one site, whereas wild type mice (n = 18) subjected to the same protocols failed to show any malignant or premalignant changes. An unexpected finding was the occurrence of esophageal SCC in 14% of IL-1α transgenic mice treated for 12 weeks, and 57% of those treated for 17 weeks. To our knowledge, this is the first reported genetic mouse model for esophageal SCC. These data conclusively demonstrate that primary inflammatory cytokines produced by epithelium play an important role in head and neck carcinogenesis. Strategies to inhibit IL-1 signaling pathways and reduce chronic inflammation may be important preventive and therapeutic modalities.

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Induction of Invasive Human Epidermal Neoplasia Resembling Squamous Cell Carcinoma (SCC) by Defined Genetic Elements Ras and CDK4

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Ras acts in combination with other unknown proteins to induce uncontrolled cell division and is the most commonly mutated proto-oncogene in human cancers. Gene expression profiling on SCCs from 12 patients demonstrated induction of Ras targets in SCCs compared to their clinically normal skin, suggesting that Ras in concert with other defined oncoproteins can directly convert normal epidermis to invasive neoplasia. To test this, we activated Ras signaling in human epidermis while simultaneously altering other regulators of cell growth, including p53, CDK4 and hTERT. Using high efficiency retroviral transductions in primary keratinocytes, we expressed active RasV12 and these regulators either singly or in combination via multicistronic retrovectors and used these cells to regenerate human skin on SCID mice [n = 4–7 per group]. Ras alone leads to complete cell cycle arrest within 48 h along with induction of the CDK inhibitor (CKI) p21 while Ras-p53W248, Ras-hTERT and CDK4 alone fail to induce epidermal neoplasia. In contrast, within 3 weeks Ras-CDK4 produced large skin tumors in 7/7 mice with histologic features of SCC, including deep invasion through fat to underlying muscle. Ras-CDK4 tumors, similar to human SCC, express increased levels of VEGF with neoangiogenesis, elevated MMPs 1/3 and decreased E-cadherin. Ras-CDK4 cells up-regulate p21 at comparable levels to Ras alone but are resistant to the mid-G1 CKIs p15 and p16, suggesting that increased resistance to CKIs rather than a failure to induce them contributes to Ras-CDK4 neoplasia. Cyclin D1 was necessary but not sufficient for Ras-CDK4 neoplasia as CDK4-D1 failed to induce tumors [n = 4 mice] while an antisense D1 retrovector that suppressed D1 tissue protein expression abolished Ras-CDK4 neoplasia [n = 4]. CDK4 synergy with Ras is dependent on intrinsic CDK4 kinase function as the kinase-dead N158 CDK4 point mutant failed to induce neoplasia when coexpressed with Ras [n = 4]. These data identify Ras and CDK4 as capable of converting normal human epidermal tissue into invasive neoplasia and indicate that functional CDK4-D1 is necessary for this process.

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Transfection of CHO Cells with Human EGFR Mutants to Elucidate EGFR Signaling Pathways in Wound Healing

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The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases is comprised of four closely related transmembrane proteins that bind multiple ligands of the EGF family. Ligand binding triggers numerous downstream signaling pathways resulting in a host of biological responses including proliferation, differentiation, migration and apoptosis. The aim of these initial studies was to create cell lines containing various EGFR mutants that can be utilized to evaluate the divergent EGFR signaling pathways following wounding. Chinese hamster ovary-K1 (CHO-K1) cells possess no detectable EGFR. We transfected CHO-K1 cells with either wild-type hEGFR or various defined hEGFR mutants with altered cytoplasmic domains. Stable transfectants were generated via multiple rounds of limiting dilution. Transfection efficiency was evaluated by Northern hybridization as well as FACS. Northern analysis revealed either normal or mutant hEGFR transcript present in all transfected cell lines. Transfected hEGFR mRNA expression levels were slightly less in CHO-K1 cells compared to that of adult normal human epidermal keratinocytes. Wild type CHO-K1 cells expressed no EGFR mRNA. FACS analysis showed detectable levels of either transfected normal or mutant hEGFR on the surface of the CHO-K1 cells. *In vitro* scratch assays were utilized to compare biological wounding responses of transfected CHO-K1 cells with that of wild type cells. Following overnight serum and growth factor depletion, postconfluent transfected CHO-K1 cultures were wounded and treated with either basal media or with basal media plus 10 mM EGF. Depending on the mutant, addition of EGF induced an increase in either cell proliferation or migration vs. that of untreated transfectants. In addition, transfected cells had a survival advantage over wild type cells both in the presence and absence EGF or serum. In conclusion, our initial results suggest that the pleiotropic response of hEGFR to various ligands is mediated through different domains of the cytoplasmic portion of the molecule. We are further investigating the specific signal transduction pathways induced by the multiple genetically distinct ligands that activate the hEGFR during the course of normal cutaneous wound healing.

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MIB-1 Proliferative Activity and Mutant p53 Expression in Cutaneous Squamous Neoplasms: A Prospective Evaluation of 117 Cases

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Background: Accurate assessment of cutaneous squamous neoplasms is essential in the effective management and treatment of these lesions. Molecular markers of cell proliferation, such as MIB-1, and mutant p53 expression have been associated with progression to carcinoma and may be helpful as an adjunct to histomorphology in assessing cutaneous squamous neoplasia. To date, prospective evaluation using a large patient cohort has not been undertaken. Aim: To prospectively assess the utility of MIB-1 and mutant p53 immunoreactivity in the evaluation of cutaneous squamous neoplasms. Methods: 117 patients with squamous cell carcinoma (SCC; n=26), in-situ squamous cell carcinoma (SCC I-S; n=45) and actinic keratoses (AK; n=46) were evaluated prospectively (6 months). A benign control group was concurrently evaluated (n=9; seborrheic keratoses, verruca vulgaris and pseudoepitheliomatous hyperplasia) . MIB-1 (AMAC; dilution-1:10) and p53 (DAKO; clone-DO7; dilution-1:10) immunostaining of paraffin-embedded formalin-fixed tissue sections was undertaken using a commercially available autoimmunostainer (Ventana ES). Semi-quantitative assessment of nuclear immunoreactivity within lesional keratinocytes was performed in all cases (<25%, 25-50%, 51-75%, >75% of cells, respectively) and blindly assessed by 2 dermatopathologists (KH,AHO). Group differences were compared using Fisher's exact test (2-sided) and sensitivity/specificity was calculated using Bayesian formulae. Results: Mean age was 69.1 years (range: 33-97 years) and 56% were male. Sites of squamous lesions included the head and neck (n=54:43%), trunk (n=19; 15%) and extremities (n=53; 42%). MIB-1 and p53 nuclear immunoreactivity (>50% of lesional keratinocytes) in SCC/SCC I-S (n=71), AK (n=46) and benign controls (n=9) was seen in 42%, 28%, 0%, and 66%, 41%, 22%, respectively. Either MIB-1 or p53 nuclear immunostaining (>50% of lesional keratinocytes) was seen in 79% (SCC/SCC I-S), 48% (AK) and 22% (benign controls), respectively. Sensitivity and specificity of p53 or MIB-1 immunoreactivity for a squamous neoplasm was 67% and 82%, respectively. Conclusion: MIB-1 and p53 immunoreactivity is moderately sensitive and specific for cutaneous squamous neoplasms and supports the use of these markers as an adjunct to histomorphology. Further evaluation of molecular markers with increased sensitivity and specificity for cutaneous squamous neoplasms is warranted.

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A Novel Colorimetric Bead-Binding Assay for Detection of Molecular Interactions

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We have developed a new technique that rapidly and reproducibly allows direct visualization of molecular interactions, including receptor-ligand binding. The technique can be easily applied to examine binding between proteins and glycoproteins, or proteins and glycolipids, including gangliosides. In this novel bead-binding assay, a target molecule is bound to colored FluoSphere beads. These coated beads are then mixed directly with molecules adherent to larger 150 μm molecules is easily detected by immunofluoresence microscopy as colored rosettes. This new technique has been used to confirm the direct interaction of the βcholera toxin with ganglioside GM1 as well as to show the binding of ganglioside GT1b with the α5 subunit of α5β1 and GM3 with the epidermal growth factor receptor. A modification of this technique, in which the coated beads are bound to protein fixed on plates, allows a quantifiable colorimetric assay of interaction. This versatile and rapid technique will have widespread applications for *in vitro* systems and may also be useful for *in vivo* analysis of binding to cell surface receptor molecules.

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Isolation of a HeLa-Derived Cell Line Resistant to E2-Mediated Growth Arrest

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There are more than 70 different types of human papillomaviruses (HPV). Certain high risk HPVs are associated with carcinomas of the mucosal and cutaneous epithelium. HeLa cells, a human cervical carcinoma cell line that contains HPV 18 DNA, express HPV E6 and E7 proteins, which neutralize tumor suppressor function. HeLa cells infected with a recombinant SV40-based virus expressing the bovine papilloma virus (BPV) E2 protein results in almost complete repression of HPV E6 and E7 expression, restoration of the p53 and Rb tumor suppressor pathways, and profound growth inhibition within 48 h of infection. After repeated infections with the recombinant virus expressing BPV E2, only one in approximately 100 000 HeLa cells proliferated to form discrete colonies. In order to characterize the nature of these cells that resist growth inhibition, we expanded individual E2-resistant colonies into cell lines and reintroduced the virus expressing BPV E2. Although many of these cell lines exhibited only partial repression of HPV E6 and E7 expression, we isolated one cell line (designated res 6B) in which HPV E6 and E7 were efficiently repressed by BPV E2. However, 48 h after E2 expression, the res 6B cell line displayed 10-fold more DNA synthesis than did infected parental HeLa cells. In summary, we isolated a HeLa-derived cell line in which BPV E2 repressed HPV E6 and E7 expression as well as in normal HeLa cells. Nevertheless, these cells demonstrated a 10-fold growth advantage as measured by E2-resistant DNA synthesis. These preliminary results indicate that the res 6B cell line is not dependent on continued HPV oncogene expression for sustained proliferation, and suggest that these cells may harbor differences in the downstream p53 and Rb tumor suppressor pathways.

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Telomere-Independent Keratinocyte Senescence Enforced by Coordinate Induction of p16INK4A and p14ARF; Spontaneous and Experimental Abrogation of this Mechanism in Culture and *In Vivo*

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Replicative lifespan in culture of fibroblasts and several other cell types is limited by telomere attrition, which triggers a p53-mediated, p21cip1-enforced senescence arrest. In contrast, for some epithelial cell types, including keratinocytes, senescence results from a mechanism that detects an aspect of cell aging other than telomere loss and which triggers p16INK4a expression. In optimized culture media, human epidermal, oral, corneal, and conjunctival keratinocytes can undergo more doublings than stem cells of these tissues are estimated to require during the human lifespan *in vivo*. Unlike their typical fate during tissue renewal, however, in culture they cease growth as senescent cells blocked in G1 with a high p16 content. We have found that abrogating the p16 block by stably transducing keratinocytes to express a p16-resistant, mutant cdk4 (cdk4R) does not detectably extend lifespan but results in the cells senescing with either a G1 or G2 DNA content, consistent with a p53-mediated, p21cip1-enforced arrest. Cells engineered to express both cdk4R and a dominant negative mutant p53 (p53DD) bypassed senescence, dividing an additional 20–30 times until halted by crisis. Keratinocytes cultured from a p16(+/−) individual senesced after a normal lifespan, but such cells transduced to express p53DD generated rare p16(−/−) variants that grew for 30 extra doublings; thus, p16 loss and expression of a p16-resistant cdk4R are equivalent phenotypically. Keratinocytes having lesions in both p16- and p53-dependent senescence mechanisms proved to have high levels of the mdm2 inhibitor p14ARF – evidence that the p53-dependent component of keratinocyte senescence is activated by p14ARF. Keratinocytes deficient in both p16 and p53 control pathways differentiated normally, as did cells we cultured from a premalignant oral epithelial lesion that had acquired p16 and p53 mutations *in vivo*. These results support a model of cancer development in which focal areas of epithelia that undergo chronic, accelerated renewal fall under selection pressure for variants able to escape both p16- and p14ARF/p53-dependent lifespan limitation. Such variants are preneoplastic because of their extended lifespan and ability to divide to crisis with potential of progressing to full malignancy.

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Pemphigus Foliaceus IgG Activates Transmembrane Desmosomal Signaling

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Pathogenic pemphigus vulgaris (PV) and pemphigus foliaceus (PF) autoantibodies bind the ectodomain of desmoglein-3 (dsg3) and desmoglein-1 (dsg1), respectively. PV and PF antibodies trigger epidermal cell-detachment (acantholysis) by unknown molecular mechanisms. Proposed mechanisms for PV and PF IgG induced acantholysis include (1) proteinase activation (2) steric hindrance, and (3) activation of transmembrane signaling that down regulates cell-cell adhesion. The purpose of this study was to test whether purified antidsg1 antibodies can activate transmembrane signal transduction cascades on normal human cultured keratinocytes in the presence of [^{32}P] H_3PO_4. PF antidsg1 antibodies were affinity-purified using a soluble fraction of the dsg1 ectodomain produced in the baculovirus system. Control keratinocyte cultures included cells incubated with no IgG or normal IgG (affinity-purified on Protein A). Keratinocyte extracts were examined by two dimensional electrophoresis and autoradiography for the presence of altered protein phosphorylation. Incubation of normal human keratinocytes with PF IgG resulted in increased phosphorylation of several proteins resolved by 2D electrophoresis when compared to control cultures. The PF IgG induced changes in protein phosphorylation are consistent with the hypothesis that the binding of PF IgG to the dsg1 ectodomain initiates a transmembrane signal transduction cascade which may contribute to the molecular mechanism of PF IgG acantholysis. Additionally, this suggests that in addition to mediating cell-cell adhesion, desmosomes function as ligand dependent transmembrane signaling receptors.

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Expression and Phosphorylation of Mitigen-Activated Protein Kinases in Melanoma Cells *In Vitro* and *In Vivo*. Evidence for a Correlation Between *In Vivo* Phosporylation and Tumor Progression

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Mitogen-activated protein kinases (MAPK) belong to large family of kinases that are regulated by a variety of extracellular signals. Although it is known that MAPK2 is activated in normal melanocytes (NHM) and melanoma cells in response to distinct growth factors and other external stimuli, little is known about the actual comparative expression and phosphorylation of MAPK1-4 in melanoma cells *in vitro* and *in vivo*. Western immunoblotting of whole cell extracts of NHM and 8 human melanoma cell lines derived from different stages of disease progression revealed that expression of MAPK1, 2 and 3 was similar between most melanoma cell lines and NHM. Expression of MAPK4 was consistently higher in all of the examined melanoma cell lines than in NHM. Phosphorylation of MAPK1/2 at Tyr204 in all melanoma cell lines, however, was markedly elevated in all melanoma cell lines as determined by immunoblotting with a phospho-specific antibody. The latter findings were corroborated by *in vitro* kinase assays demonstrating an up to 18-fold higher MAPK1/2 activity in melanoma cells as compared to NHM. In order to check if phosphorylation of MAPK1/2 at Tyr204 can also be detected in melanomas *in situ*, a total number of 73 cutaneous melanomas were evaluated by the immunoperoxidase technique. In normal human skin, MAPK1/2 phosphorylation was not detectable. In primary melanomas, in general, immunostaining correlated with tumor invasiveness, while in tumor metastases immunostaining was heterogeneous. Our data highlight the importance of MAPK in human melanomas and suggest a role of phosphorylated MAPK1/2 as a molecular correlate of tumor progression.

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CD44 Proteoglycans from Human Keratinocytes Bind to the Glycosaminoglycan Binding Domain of Amphiregulin But Not of Heparin Binding EGF

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Evidence from neutralizing antibody inhibition suggests that amphiregulin is the predominant autocrine mitogen for human keratinocytes, with HB-EGF serving a lesser role. Endogenous heparan sulfate proteoglycans such as CD44 isoforms may be obligate cofactors in their signaling through EGFR-class receptors. Exogenous heparinoids, as competitive ligands with endogenous heparan sulfates, strongly block the proliferative effects of amphiregulin, but not of HB-EGF, despite the heparin-binding properties of both. That exogenous heparinoids strongly inhibit keratinocytes thus further substantiates that amphiregulin, and not HB-EGF, is the principal autocrine factor. We hypothesized that differences in affinities of the mitogens' binding domains for specific, endogenous heparan sulfates account for the disparate effects of exogenous heparinoids. Metabolically labeled, heparan sulfate-containing CD44 proteoglycans were isolated by specific immunoprecipitation from cultured cells. Samples were chromatographed on standardized affinity columns of either gel-immobilized amphiregulin or HB-EGF peptide, the 25-mer peptide corresponding to each growth factor's putative heparinoid binding domain. CD44 proteoglycans had much stronger affinity for amphiregulin peptide than for HB-EGF peptide, as evidenced by little or no binding of any CD44 fraction to the latter. The identities of the eluants were confirmed by Western blotting with anti-CD44. These data support the hypothesis that endogenous CD44 specifically regulates the effects of amphiregulin and that HB-EGF may be regulated by cofactors other than CD44. Collectively, our results further confirm CD44-regulated amphiregulin as the principal autocrine factor in keratinocytes.

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Inactivation of O6-Methylguanine-DNA Methyltransferase (MGMT) by Promoter Hypermethylation is Associated with Loss of MGMT Protein Expression in Skin Cancer

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Defective repair of O6-methylguanine adducts is relevant to carcinogenesis since these lesions are highly mutagenic. Previous studies in human cancer have shown that one mechanism for defective repair is lack of expression of MGMT enzyme which repairs these lesions. This frequently occurs through hypermethylation of CpG sites within the MGMT promoter. To assess the role of MGMT silencing in the multistage mouse skin carcinogenesis model, methylation status of the MGMT promoter was examined by methylation specific PCR (MSP) and bisulfite-modified sequencing. The results were correlated with the expression status of the gene. Additionally, the times at which MGMT hypermethylation occurred was detected, and the relationship of MGMT hypermethylation to mutations in the ras oncogene was also examined. Among 184 skin tumors examined, 81 (44%) demonstrated MGMT hypermethylation. No significant differences were observed between MGMT methylation among papillomas and carcinomas. Hypermethylation was detected in tumors as early as 5 weeks after MNNG initiation and as early as 7 weeks after DMBA initiation. MGMT methylation was more frequent with DMBA initiation ($p = 0.006$), and methylation increased with time ($p = 0.009$). Promoter hypermethylation was also examined by bisulfite sequencing. The distribution of methylated sites was heterogeneous. A statistically significant association was observed between MGMT promoter hypermethylation and the absence of MGMT protein expression ($p < 0.0001$; Fisher's exact test). No significant association was observed between MGMT methylation and either Ki-ras or Ha-ras activation. Thus, MGMT promoter hypermethylation is an early and frequent event in squamous tumor development, but it is not linked to ras activation in this carcinogensis model. Silencing of the MGMT gene may contribute to tumor progression by increasing the frequency of mutagenic events associated with endogenous alkylation damage of DNA.

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Epidermal Growth Factor Receptor Glycosylation is Required for Ganglioside GM3 Binding and GM3-Mediated Suppresion of Activation

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Gangliosides bind to the epidermal growth factor receptor and inhibit its activation, but the mechanism of this inhibition is unknown. To address the role of receptor carbohydrates in facilitating interaction with gangliosides, we examined the ability of GM3 to bind the deglycosylated receptor and inhibit its autophosphorylation. Flow cytometry studies demonstrated that deglycosylation of the receptor did not affect its transport to the cell membrane. In contrast with the native (fully glycosylated) receptor, GM3 did not coimmunoprecipitate with the deglycosylated receptor. Using a novel colorimetric bead binding assay, GM3 was shown to bind well to the immunoprecipitated native receptor, but not at all to the deglycosylated receptor. Finally, the addition of GM3 to cells with deglycosylated epidermal growth factor receptors did not result in significant further inhibition of autophosphorylation of the receptor, despite a 10-fold decrease in phosphorylation of the native epidermal growth factor receptor by 200 μM GM3. These studies suggest that ganglioside modulates epidermal growth factor receptor activity through a direct interaction that requires receptor glycosylation, and contribute to our understanding of the role of gangliosides in cell membrane function.

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Ganglioside GT1b Induces Apoptosis of Keratinocytes by Modulation of Protein Kinase B/Akt Phosphorylation through the Integrin Linked Kinase Pathway

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GT1b comprises 8% of the total membrane ganglioside content of keratinocytes and keratinocyte-derived SCC12 cells. We have previously shown that GT1b is able to bind directly to the α5 subunit of α5β1 integrin through carbohydrate–carbohydrate interactions, thereby blocking the binding of α5β1 to fibronectin (FN) with resultant apoptosis. Integrin linked kinase (ILK) and protein kinase B (PKB)/Akt are serine/threonine protein kinases that are thought to modulate keratinocyte cell survival and apoptosis. We hypothesized that GT1b triggers keratinocyte apoptosis through modulation of ILK activity and its downstream substrates PKB/Akt and Caspase-9. Western blotting techniques were used to compare the alterations in phosphorylation of β1 integrin, ILK, PKB/Akt, Bad, and Caspase-9 among SCC12 cells, SCC12 cells pretreated with specific anti-GT1b antibodies, cells supplemented with GT1b, and SCC12 cells depleted of all gangliosides by gene-modulated overexpression of a ganglioside-specific sialidase. Our studies show that ganglioside depletion increases phosphorylation of Bad, a downstream substrate of PKB/Akt, and inhibits Caspase-9, thus preventing apoptosis, even when stimulated by serum deprivation, acetylsalicylic acid, or staurosporine. In contrast, supplemental GT1b suppresses phosphorylation of Bad, and activates Caspase-9, leading to cell cycle arrest and initiation of apoptosis. Plating of ganglioside-depleted SCC12 cells on a FN matrix results in stimulation of β1 integrin phosphorylation, ILK phosphorylation, and PKB/Akt activity at both Ser-473 and Thr-308 sites. The antiapoptotic effect is eliminated when the ganglioside-depleted SCC12 cells are transiently transfected with PKB/Akt cDNA containing mutations at both the Ser-473 and Thr-308 phosphorylation sites. The addition of GT1b to SCC12 cells inhibits phosphorylation of β1 integrin and ILK, whereas anti-GT1b antibodies stimulate their phosphorylation; blockade or addition of other gangliosides does not influence phosphorylation of β1 integrin or ILK. These studies suggest that GT1b modulation of keratinocyte cell cycle regulation and survival are mediated through PKB/Akt inhibition via the integrin linked kinase signaling pathway.

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Site-Specific Suppression of PKB/Akt Phosphorylation by Keratinocyte Ganglioside: Relation to Apoptotic Potential

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Gangliosides have been implicated in the regulation of cell proliferation, differentiation, adhesion and migration, and apoptosis. The ganglioside content of keratinocyte membranes includes GM3 (62.9%), 9-O-acetylGD3 (16.9%), GD3 (13.7%), and GT1b (6.5%). We have previously shown that GT1b inhibits the adhesion of keratinocytes on fibronectin (FN) through binding to and inhibition of α5β1 activity; GM3 binds to the epidermal growth factor receptor to inhibit cell proliferation. We studied the effects of these keratinocyte gangliosides on cell apoptosis and on apoptosis-related signal transduction, including phosphorylation of protein kinase B (PKB)/Akt, a serine/threonine protein kinase that is thought to modulate cell survival and apoptosis. Depletion of gangliosides in keratinocyte-derived SCC12 cells by gene-modulated overexpression of a ganglioside-specific sialidase resulted in complete resistance to apoptosis in cells plated on a FN matrix, even when subjected to serum deprivation, or treatment with acetylsalicylic acid or staurosporine. In contrast, SCC12 cells supplemented with GT1b became apoptotic when plated on FN; this effect was not noted when GM3 was substituted for the GT1b. Treatment of SCC12 cells with anti-GT1b antibodies, but not with anti-GM3 antibodies resulted in reduced ability to apoptose but not complete resistance to apoptosis. In SCC12 cells depleted of gangliosides, protein kinase B (PKB)/Akt phosphorylation was markedly increased at both serine 473 and threonine 308, resulting in inhibition of Caspase 9 activation. Blockade of GT1b function by anti-GT1b antibodies increased and treatment with GT1b virtually eliminated phosphorylation of PKB/Akt; however, modulation of GT1b function affected only phosphorylation of the Ser-473 site. Blockade of GM3 function by anti-GM3 antibodies and treatment of SCC12 cells with GM3 caused increased and decreased phosphorylation only of the Thr-308 phosphorylation site. Consistently, inhibitors of EGFR-associated tyrosine kinase and PI3 kinase inhibitor inhibited phosphorylation only at the Thr-308 site, and not at the PKB/Akt Ser-473 phosphorylation site. These studies suggest that ganglioside regulation of induced keratinocyte apoptosis is predominantly modulated at the PKB/Akt Ser-473 phosphorylation site, but that increased phosphorylation of the Thr-308 site is necessary for complete ganglioside-induced resistance to apoptosis.

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Gangliosides Modulate Keratinocyte Adhesion, Spreading and Migration by Both Integrin- and EGFR-Mediated Signaling Pathways

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Keratinocyte gangliosides modulate both the epidermal growth factor receptor (EGFR)-and α5β1 integrin-mediated signaling pathways. Focal adhesion kinase (FAK) is a widely expressed cytoplasmic tyrosine kinase that colocalizes with integrins, whereas Ras and MAP kinase (MAPK) are downstream components common to both EGFR- and integrin-mediated signaling pathways. We hypothesized that keratinocytes depleted of gangliosides would show increased adhesion, spreading and migration on a fibronectin (FN) matrix after exposure to EGF. We proposed that the mechanism of the effect of ganglioside depletion requires modulation of FAK activity through both the EGFR- and integrin-mediated signaling pathways. Keratinocyte-derived SCC12 cells were depleted of ganglioside by gene-mediated overexpression of a ganglioside-specific sialidase. Adhesion, spreading and migration on a FN matrix were increased in response to EGF in sialidase-transfected cells, but not in parental SCC12 or mock-transfected cells. In parental SCC12 cells treated with antibodies directed against GM3 or GT1b gangliosides, adhesion, spreading, and migration were also increased in response to EGF. Concomitantly, FAK and MAP kinase phosphorylation were greatly increased in these ganglioside-depleted or ganglioside-blocked cells plated on FN, but not in cells plated on plastic or polylysine-coated plates. Antibodies directed against ganglioside 9-O-acetylGD3 had no effect on cell function or on FAK or MAPK activation. Both EGFR-associated tyrosine kinase inhibitor and PI3 kinase inhibitor decreased cell adhesion, spreading, and migration, and suppressed the FAK and MAPK activation in these ganglioside-depleted keratinocytes. Ras farnesyltransferase inhibitor also block the MAP kinase activation. We conclude that gangliosides regulate cell adhesion and migration by modulating FAK phosphorylation through both EGFR-mediated and integrin-mediated signal pathways in SCC12 cells. In addition, the FAK signaling pathway affected by gangliosides requires Ras activation for MAP kinase phosphorylation and modulation of cell behavior on a FN matrix.

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Negative Regulation of the Akt Cell Survival Pathway by the Protein Kinase C Signal Transduction Pathway

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Isoforms of protein kinase C (PKC), a family of 11 serine/threonine kinases, are important mediators of keratinocyte proliferation, differentiation, and apoptosis. While certain downstream elements of the PKC pathways have been identified, a complete analysis will be required to identify novel targets for pharmaceuticals that would influence skin biology more specifically. One potential downstream effector of apoptosis is protein kinase B (PKB or Akt), an important mediator of cell survival. In mouse keratinocytes activation of PKC by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) reduces Akt phosphorylation on Ser 473 (P-Akt), the active form of PKB. The reduction of Akt phosphorylation is prevented by treating keratinocytes with the specific PKC inhibitor GF109203X prior to treatment with TPA. Furthermore, pretreatment of mouse keratinocytes with TPA attenuates Akt phosphorylation induced by insulin-like growth factor-1, epidermal growth factor or serum. Inhibitors of protein phosphatases, okadiac acid and calyculin A, increase P-Akt and prevent the reduction of P-Akt caused by TPA treatment. Thus, activation of PKC signal transduction causes inactivation of Akt, likely by phosphatase activation. This interaction of PKC and Akt signaling could be associated with apoptosis in keratinocytes exposed to PKC activators or PKC mediated cell death associated with terminal differentiation.

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Cell Cycle Dysregulation and Apoptosis of Human Epidermoid Carcinoma A431 Cells by *trans*-Resveratrol

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Resveratrol (*trans*-3, 4′, 5,-trihydroxystilbene), a potent antioxidant phytoalexin, is known to be present in grapes, nuts, fruits and red wine. Recent *in vitro* and *in vivo* laboratory studies have suggested that resveratrol possesses cancer chemopreventive properties against many cancers including chemically induced skin carcinogenesis in mouse model. The mechanism(s) by which resveratrol imparts skin cancer chemopreventive effects is not clearly defined. In this study, employing human epidermoid carcinoma (A431) cells, we evaluated the involvement and mechanism of cell cycle dysregulation in antiproliferative properties of resveratrol. Resveratrol (1–50 μM) treatment of A431 cells resulted in a dose-dependent (i) inhibition of cell growth as shown by MTT assay (ii) G1-phase arrest of the cell cycle as shown by DNA cell cycle analysis, and (iii) induction of apoptosis as assessed by enzyme-linked immunosorbent assay. The immunoblot analysis revealed that resveratrol treatment causes a dose- as well as time-dependent (i) induction of WAF1/p21 (ii) decrease in the protein expressions of cyclin D1, cyclin D2, and cyclin E, and (iii) decrease in the protein expression and kinase activities of cyclin dependent kinase (cdk) 2, cdk 4 and cdk 6. These data establish the involvement of cki-cyclin-cdk network during antiproliferative potential of resveratrol in A431 cells. Because (i) retinoblastoma (pRb) and E2F family transcription factors are important for the regulation of G1-S transition in the cell cycle, and (ii) the activity of the pRb is regulated by cyclin-cdk network-mediated phosphorylation, we also evaluated the effect of resveratrol on pRb-E2F/DP machinery. Immunoblot analysis demonstrated that resveratrol treatment results in a dose- and time-dependent (i) decrease in the hyper-phosphorylated form of pRb with a relative increase in hypo-phosphorylated pRb, and (ii) down-regulation in the protein expression of E2F (1 through 5) family transcription factors, and their heterodimeric partners DP1 and DP2. Taken together, our study suggests that resveratrol, via modulating cki-cyclin-cdk network and pRb-E2F/DP machinery, causes an arrest of cell cycle at G1-phase, which is an irreversible process that ultimately results in an apoptotic death of cancer cells. Because in recent years, the cell cycle inhibitors are being appreciated as new generation of anticancer drugs, we suggest that resveratrol may be a potent chemopreventive agent for skin cancer.

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Loss of Prostaglandin E (PGE) Receptor Subtype EP2 in HaCat Keratinocytes Results in an Invasive Phenotype and Decreased Expression of the Focal Adhesion Protein Paxillin

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Increased prostaglandin production and decreased PGE2-coupled cAMP responses are associated with epithelial malignancies. We transfected a spontaneously immortalized but nontumorigenic human keratinocyte cell line, HaCat, with sense and antisense constructs for the PGE receptor subtype EP2. EP2 expression was documented by immunoblot. EP2 receptors are coupled to activation of adenylyl cyclase. Utilizing a collagen raft made with human diploid fibroblasts (skin equivalent), 4 of 6 EP2 antisense clones showed a high degree of invasion into the ''dermal'' compartment and marked cellular atypia. These features were not seen in vector controls and sense transfectants. Invasion with cellular atypia in skin equivalents is typical of squamous cell carcinomas and virally transformed keratinocytes. Disruption of cellular adhesion to matrix via integrin-focal adesion complexes is known to be associated with an invasive phenotype. By Western blot, EP2 antisense clones showed decreased expression of paxillin, a critical component of focal adhesion assemby. HaCat cells synthesize PGE2. Coculture of keratinocytes with fibroblasts is known to synergistically induce PGE2 formation. EP2 receptors are known to undergo ligand-dependent down-regulation. We therefore examined the effect of ligand-induced receptor down-regulation on paxillin expression. Antisense clones were more sensitive to receptor down-regulation using a selective EP2 receptor agonist. Receptor down-regulation resulted in further loss of paxillin expression. This data suggests that loss of EP2 receptors represents a mechanism for neoplastic progression to an advanced invasive phenotype. One potential mechanism for loss of these receptors is down-regulation by the marked increase in PGE2 produced by malignant tissues.

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Expression of Novel Germ Cell Gene SPAF at Conversion to Squamous Cell Carcinoma

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Ectopic expression of primitive stem cell-specific genes in adult cells is an attractive conceptual mechanism for malignant conversion. In search of altered gene expression during multistage carcinogenesis in a clonal epidermal cell model by RNA differential display, we identified a novel spermatogonia associated factor (SPAF) that is altered and overexpressed in a poorly differentiated squamous cell carcinoma compared to normal and initiated progenitor cells. Sequence analysis reveals that SPAF protein contains two ATPase modules, which classifies it as a novel member of the AAA-protein family (ATPase associated with diverse activities). The sequence similarities between SPAF and other members containing two ATPase modules range from 50%–66% (identity of 25%–45%) over the whole length of the proteins. Using mouse SPAF cDNA as query, we retrieved a sequence fragment of candidate human SPAF that was originally cloned from a human kidney tumor. This is consistent with our finding that mouse SPAF is highly expressed in progenitor cells in testis and abnormally up-regulated in adult epithelial malignancy. The presumptive human SPAF cDNA was partially cloned by means of RACE PCR from normal human testis and sequenced. This 1183 bp partial cDNA showed 81% similarity and 78% identity at the protein level with murine SPAF, which is significantly higher than with other human AAA-proteins, indicating that the cDNA we cloned most likely represents the human counterpart of murine SPAF. The availability of mouse SPAF cDNA provided us opportunities to study its normal functions as well as potential roles in tumorigenesis. In view of a mitochondrial-localization-like signal, sequence similarities to membrane-associated proteins, ATP binding properties, and intracellular expression patterns in testis, we speculate that SPAF protein may be involved in morphological and functional mitochondrial transformations for adaptation to decreased oxygen tension during spermatogenesis. Ectopic expression of the SPAF gene in malignant tumors may facilitate the adaptation of cancer cells to the hypoxic conditions common in solid tumors. Further analysis of SPAF by generation of SPAF knock-out mice and *in vivo* tumorigenesis will provide direct evidence regarding its role in germ cell development and malignant transformation.

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Hair Cycle-Dependent Expression Patterns of STAT-3 in Mouse Skin

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STAT-3 is a member of a family of Signal Transducers and Activators of Transcription that mediate the biological actions in response to cytokines including interleukins and EGF. Recently, it was shown that STAT-3 plays a critical role in early mammalian embryonic development and is essential for hair cycle initiation and skin wound healing possibly by triggering keratinocyte migration (*EMBO J* 18:4657, 1999). To further characterize the role of STAT-3 in hair follicle biology, we have assessed its expression in the back skin of C57BL/6 mice during defined stages of hair follicle morphogenesis and cycling. Immunohistochemistry revealed exclusively cytoplasmic localization of STAT-3. The early stages of hair follicle morphogenesis were characterized by the complete absence of STAT-3 in hair follicle epithelia, consistent with normal first hair pelage in STAT-3 deficient mice. STAT-3 expression occurred during the IRS formation in its middle portion and remained constant until the onset of catagen. The catagen-telogen follicles were STAT-3 negative. Interestingly, during the telogen-anagen transition, a few (usually 1–3) STAT-3 positive cells were localized to the lowermost portion of the hair follicle, in immediate contact with dermal papilla. In early anagen, STAT-3 positive cells represented only a minor part of the lowermost downward growing hair follicle. In anagen III, with the initiation of hair matrix formation, STAT-3 positivity in the lower hair follicle disappeared. Similar to morphogenesis, during anagen the STAT-3 positive cells formed a thin (1–2 cells) ring within the middle IRS, at the level where the IRS loses its connection with the hair shaft cuticle. The occurrence of STAT-3 positive cells during anagen initiation in immediate contact with dermal papilla, and restriction of its expression to selected cells of the rapidly proliferating epithelial bud, suggests a critical role of STAT-3 in anagen induction. These findings are consistent with a failure of anagen induction in the skin of STAT-3 deficient mice (*PNAS* 97:13824, 2000). The role of STAT-3 expression in the middle portion of hair follicle epithelial sheath during mature anagen remains to be determined.

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The Epidermal Product of the Shh Target Gene BEG4 Predicts a Conserved Proline-Rich Protein that Regulates Growth Factor Signaling with the Cytoskeleton

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Sonic hedgehog (Shh) target gene activation plays a central role in hair follicle morphogenesis and Basal cell carcinoma (BCCs) formation. Using a microarray screen for genes expressed in BCC, we have identified a novel gene BCC-enriched gene 4 (BEG4). BEG4 is normally expressed in Shh responsive cells in the hair follicle and in the epithelium of BCCs, but not in interfollicular epidermis or squamous cell carcinomas. BEG4 is ectopically induced with ectopic Shh expression in transgenic animals. BEG4 is expressed in fibroblasts from patched 1 (ptc) −/− embryos, but no RNA accumulation is detected in ptc+/− or ptc−/− cells overexpressing wild type ptc, supporting BEG4's role as a Shh target gene. Previously published transcripts predict a 356 aa protein on human chromosome 8. However, we have found the major transcript in skin comes from an alternative promoter that gives rise to an 755 aa protein with a conserved coiled-coil domain. This longer BEG4 transcript predicts a novel proline-rich protein that coordinates growth factor signaling with the cytoskeleton.

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Inhibition of TGFβ Signaling in Keratinocytes Activates ERK Through a Smad and MEK Independent Pathway

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Recent studies from a number of laboratories have shown shown significant interactions between the TGFβ and mitogen activated protein kinase (MAPK) signaling pathways. To investigate what role autocrine TGFβ signaling has in regulating the MAPK pathway in primary keratinocytes, we infected cells with an adenovirus expressing a dominant negative TGFβ type II receptor (AdTGFβTR), and measured activation of various MAPK pathways with phosphorylation specific antibodies. Infection of keratinocytes with this adenovirus completely blocks response to exogenous TGFβ1. As early as 6 h after infection with AdTGFβTR, and lasting for at least 24 h, there was an increase in phosphorylation of ERK1 and ERK2. By 48 h after infection there was a significant increase in apoptosis in the AdTGFβTR infected keratinocytes. Infection with a control adenovirus AdGFP or mock infection had no effect on ERK activation, and did not cause significant induction of apoptosis. There was no alteration in p38 or JNK phosphorylation or protein levels, indicating specificity of ERK activation by AdTGFβTR. There was no increase in phosphorylation of the upstream activators of ERK: MEK1/2 and c-RAF, as well as MEK3/6. Surprisingly, treatment of AdTGFβTR infected keratinocytes with the MEK1/2 inhibitors PD90985 or UO126, at concentrations sufficient to completely block EGF-induced ERK activation, caused superactivation of ERK. Pretreatment of keratinocytes with TGFβ1 caused a slight inhibition of EGF-induced activation of ERK1/2. To test if ERK activation was dependent on Smad signaling, we infected keratinocytes with a Smad 7 adenovirus which completely blocks TGFβ1 induced gene expression through inhibition of R-smad phosphorylation by the activated type I receptor. In these Smad7 infected keratinocytes there was no activation of the ERK, JNK or p38 signaling pathways. These results suggest that AdTGFβTR blocks a TGFβ1 regulated ERK phosphatase, that is itself inhibited by MEK, and that this signaling pathway is independent of the Smad pathway.

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The TIG3 Tumor Suppressor Protein is a Potent Negative Regualtor of Keratinocyte Proliferation

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Retinoids (vitamin A and related ligands) cause profound biological changes in keratinocytes. *In vitro*, retinoids coordinately regulate expression of a variety of keratinocyte genes, and suppress keratinocyte proliferation. *In vivo*, retinoids can cause epidermal thickening, parakeratosis, and altered differentiation marker expression. The underlying mechanisms that mediate these responses are not well understood. TIG3 is a novel, recently discovered gene that was isolated from retinoid-treated psoriatic keratinocytes. TIG3 levels are increased by retinoids and this retinoid-dependent increase is correlated with reduced cell proliferation. The gene encodes a 164 amino acid tumor suppressor that is a member of the H-rev protein family of growth regulators. In spite of its potent biological activity, the mechanism of TIG3 action is not understood. In the present study, we examine the ability of TIG3 to regulate normal human keratinocyte proliferation. Adenovirus-mediated expression of TIG3 in keratinocytes produces a TIG3 concentration-dependent inhibition of cell proliferation. Thus, TIG3 can suppress keratinocyte proliferation alone, in the absence of other retinoid-associated effects. TIG3 also produced a remarkable morphological change, causing the keratinocytes to flatten and become highly adherent. Immunoflourescence studies shows that TIG3 localizes in a perinuclear region. A mutant form of TIG3, TIG1-134, that lacks the hydrophobic carboxyl terminus, does not inhibit cell proliferation. This suggests that the carboxy-terminal hydrophobic domain, which is conserved among members of the H-rev family, is required for optimal activity. Thus, our results indicate that TIG3 is a potent suppressor of keratinocyte cell proliferation, and suggest that TIG3 is likely to be an important mediator of retinoid-dependent growth suppression.

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Endogenous Activation of Protein Kinase C Regulates Proliferation and Differentiation in HaCaT Keratinocytes

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We have previously shown that human HaCaT keratinocytes express numerous protein kinase C (PKC) isoforms (the "conventional" cPKCα, β, and γ; the "novel" nPKCδ, ε, η, and θ; and the "atypical" aPKCζ), and that the activation of PKCs by phorbol esters inhibits proliferation and induces terminal differentiation. In this study we investigated the endogenous role of the PKC system in the regulation of the above processes using PKC-specific inhibitors. Differentiation was measured by either Western blot analyses of differentiation markers (the granular cell marker keratin 10 and the spinous cell marker involucrin) or by measuring intracellular calcium concentration ($[Ca^{2+}]_i$), whereas cellular proliferation was assessed by a bromo-deoxyuridine cell proliferation ELISA. Inhibition of cPKC and nPKC isoforms by GF109203X did not modify cellular proliferation. However, GF109203X differentially altered the expression of the differentiation markers; whereas the expression of the early (granular cell) marker keratin 10 was not affected, the inhibitor decreased the level of the late-terminal (spinous cell) marker involucrin in a dose-dependent manner. In addition, selective inhibition of the cPKC isoforms by Gö6976 markedly inhibited cellular proliferation in a dose-dependent fashion. Furthermore, Gö6976, similarly to that seen in the case of GF109203X, decreased the expression of involucrin without significantly modifying the level of keratin 10. Finally, neither inhibitor modified the $[Ca^{2+}]_i$ of the cells. These data indicate that the endogenous activation of PKC isoforms regulates proliferation and the expression of differentiation markers (but not $[Ca^{2+}]_i$) in HaCaT keratinocytes. In addition, our data strongly argue for the antagonistic roles of the cPKC and nPKC isozymes in the regulation of proliferation, and for the specific roles of the cPKC isoforms in the regulation of terminal differentiation of these cells.

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Abrogated Production of Chemokines by HPV16-Harboring Tumor Cells

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Recruitment of leukocytes to the site of HPV infection is necessary for eradication of transformed cells and lesion regression, which may depend, in part, on local production of chemokines. The aim of the study was to evaluate production of selected CXC (IL-8 and GROα) and CC (MCP-1 and RANTES) chemokines by nontumorigenic HaCaT keratinocytes and HPV16-harboring Skv, CaSki, and SiHa tumor cells. Transplantation of these cells into nude mice revealed that HaCaT cells induced strong local inflammatory reaction and were gradually destroyed, whereas HPV16-harboring tumor cells did not evoke significant infiltrates and formed rapid growing tumors. Chemotactic activity of culture supernatants generated by the tested cell lines was evaluated toward human neutrophils and monocytes using Transwell(r) (Costar) migration system. Chemokine production was evaluated by specific enzyme-linked immunosorbent assays, and chemokine mRNAs were detected by ribonuclease-protection assay (RPA) using specific RNA probes. HaCaT cells exerted strong chemotactic activity toward both neutrophils and monocytes, and released relatively high amounts of the respective chemokines. Chemotactic activity of HPV16-harboring tumor cells toward neutrophils and monocytes was significantly decreased, and production of the respective chemokines was decreased or absent. Production of chemokines by HaCaT cells could be significantly up-regulated by recombinant human TNFα (rhTNFα). However, TNFα only partially stimulated chemokine production by HPV16-harboring cells. The study showed that cell transformation by oncogenic HPVs is associated with abrogation of chemokine production that may be an important factor enabling unopposed growth of HPV-associated lesions.

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Impact of Specific Receptor Tyrosine Kinases on Ras in Epithelial Cells

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Ras proteins function as molecular switches downstream of receptor tyrosine kinases (RTKs) to transmit signals regulating cell growth and differentiation. A number of RTKs are capable of activating Ras in various cell types and tissues, however, not all of them function in epidermis. It has been demonstrated that targeted disruption of the RTKs EGFR, PDGFRa and IGF1R leads to major defects in epidermal growth and differentiation. To determine the impacts of these RTKs on epidermal Ras, we stimulated quiescent primary keratinocytes with individual growth factors and examined Ras activation and MAP kinase activity. Active GTP-bound Ras protein can be pulled down from total cell extract by a GST fusion to the Ras-binding domain of Raf1 (aa 51–131) and quantitated as a percentage of total Ras, thus providing a direct biochemical measure of the degree of Ras activation. EGF activates Ras and MAP kinases at concentrations as low as 2 ng per ml while PDGF and IGF1 function at 10-fold higher concentrations. Activation of p42/p44 MAPKs does not completely correlate with the activation of Ras upon stimulation by different growth factors. A 10% increase in active GTP-bound Ras induced by EGF causes a 40% induction of active MAPK, however, a 35% increase of active Ras by IGF1 only generates a 20% induction in active MAPK, indicating that these RTK ligands exert differential effects on downstream Ras effectors. Both the general tyrosine kinase inhibitor genistein and specific RTK inhibitors block Ras and downstream MAPK activation. While all 3 Ras isoforms, H-Ras, K-Ras and N-Ras, are expressed in epidermis, they respond differently to upstream RTKs. For example, N-Ras is strongly activated by IGF1R while H-Ras is unaffected. In addition we are undertaking studies with EGFR, PDGFR and IGF1R knockout mice, which will allow us to study activation of Ras and downstream effectors in the context of selective absence of specific RTKs. These data indicate that Ras can be activated by 3 RTKs of functional importance in epidermis in a tyrosine kinase-dependent fashion, establish that different RTKs exert differential impacts on specific Ras downstream effectors and suggest that different RTKs may mediate their effects via distinct isoforms of Ras.

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Identification and Characterization of Initiation-Associated Genes in a Clonal Epidermal Model of Carcionogenesis

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Our laboratory has developed a strategy to identify and functionally test candidate-initiation genes. The steps are to (1) detect candidate genes by differential display or microarray comparison of nontransformed progenitor keratinocytes (291), 7,12-dimethylbenz[α]anthracene (DMBA)-initiated cells, benign papilloma cells, and squamous cell carcinoma derivatives (2) test transforming function of candidate genes *in vitro* by measuring induction of resistance to Ca^{2+}-induced terminal differentiation and *in vivo* by skin-grafting and tumorigenesis, and (3) determine relevance and potential significance of candidate genes by testing frequency of altered expression in independently derived tumors. As an example, one gain of function at initiation, 2G2, was detected by differential display representing 5% of cellular mRNA compared to 17 changes at malignant conversion. Northern blotting confirmed elevated 2G2 expression in 3 independently initiated clones. The full-length 2G2 cDNA was cloned from a testis cDNA library and the 5-prime end was obtained by RACE PCR. At the deduced amino acid level, 2G2 showed 95% identity to human protein HT2A and includes motifs found in oncogenes and transcription factors: a ring finger domain, cys/his cluster, and coiled-coil region. Initiating ability was tested using retroviral infection of 291 cells to introduce 2G2, green fluorescent protein-GFP, or known initiator ras. 2G2-expressing colonies resistant to 1.4 mM Ca^{2+}-induced terminal differentiation were increased 2-fold (17% transformation frequency) compared to GFP (8%) but less than ras (33%) when treated with 12-o-tetradecanoylphorbol-13-acetate (TPA). Exogenous expression of the introduced genes was confirmed by Northern blotting. The role of 2G2 in initiation is subject to verification by *in vivo* assays of papilloma formation from 291 cells infected with retrovirus containing 2G2, GFP, or ras followed by skin-grafting to athymic nu/nμ mice and treatment with TPA. Quantitative real-time-PCR is being used to determine 2G2's prevalence in independently derived mouse and human squamous cell carcinomas. Microarray analysis will be used to identify genes responsive to 2G2 to test the hypothesis that 2G2 is a transcription factor for initiation and/or promotion genes. The current strategy to study initiation genes may provide new leads for the development of early diagnostic tests, preventive measures, and treatments for human cancer.

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Insulin Induced PKCδ Activity Mediates STAT3 Transcriptional Activation in Keratinocyte Proliferation

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STAT (Signal Transducers and Activators of Transcription) proteins are a family of transcription factors recruited by a variety of cytokines and growth factors. Among the seven known STAT family members STAT3 is unique. Targeted disruption of STAT3 but not other STAT family members leads to early embryonic lethality. Specifically, when STAT3 was conditionally ablated in skin, skin remodeling was severely disrupted. Upon activation, STAT proteins form homo or heterodimers, translocate to the nucleus and bind to DNA response elements of target genes to induce transcription. To elucidate the pathway by which STAT3 activation is regulated in skin we utilized a model of skin keratinocytes in culture. It was found that in keratinocytes, PKCdbut not other PKC isoforms expressed in skin (PKCs α, ζ, η and ε) is constitutively associated with STAT3. Furthermore, insulin regulates phosphorylation and activation of STAT3 via specific activation of PKCδ. As early as 5 min following insulin stimulation, PKCδ but not PKCs α, η, ε and ζ is specifically activated. PKCδ activation is associated with induction of serine and tyrosine phosphorylation of STAT3 bound to PKCδ, which is followed by STAT3 nuclear translocation and transcriptional activation. Overexpression of adenovirus PKCδ, but not of adenoviral constructs of PKCs α, η, or ζ induced STAT3 association with PKCδ and STAT3 serine phosphorylation. Furthermore, inhibition of PKCδ activity by a pharmacological inhibitor, rottlerin, or by overexpressing a dominant negative PKCδ mutant abrogated insulin induced STAT3 phosphorylation and nuclear translocation. Finally, overexpression of a dominant negative PKCδ mutant inhibited keratinocyte proliferation induced by overexpression of STAT3. Overall, these results suggest a role for insulin induced PKCδ activity in transcriptional activation by STAT3 in skin keratinocyte proliferation. Further understanding of the role of insulin in mediating PKC signaling and transcriptional activation could shed light on the unique role of insulin in proliferation and differentiation of skin keratinocytes.

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Mitogenic Signaling Pathways Required for Induction of the Novel Immediate Early Gene IEX-I by Epidermal Growth Factor Receptor (EGFR)

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Cells from organs and tissues with higher proliferative rate express higher levels of the novel Immediate Early Gene, IEX-I. Additionally, overexpression of IEX-I in cultured cells leads to an increased rate of proliferation. Transcription of IEX-I is rapidly and transiently induced in a variety of cells upon stimulation with appropriate growth factors. Activation of the Epidermal Growth Factor Receptor (EGFR) in cultured keratinocytes leads to both induction of IEX-I mRNA transcription and enhanced proliferation. However the exact nature of the proliferative signals mediating EGFR induction of IEX-I mRNA is not clearly understood. Both Mitogen Activated Protein Kinase (MAPK) and Phosphatidylinositol 3-Kinase (PI3K) pathways have been implicated in regulation of cellular proliferation in multiple cell types. Here we examined whether these known mitogenic signaling pathways play a role in regulation of IEX-I mRNA induction by EGFR. We find that pretreatment of keratinocytes (HaCaT) with EKI 785, a specific inhibitor of EGFR, completely abolishes EGF-induced IEX-1 transcription. Inhibition of either MAPK pathway alone using MEK inhibitor PD98059 or PI3K pathway alone using wortmannin had no effect on EGFR-mediated IEX-I induction. However, concomitant pharmacologic inhibition of both MAPK pathway and PI3K lead to a complete abrogation of IEX-I mRNA induction in response to EGF stimulation. This suggests essential but overlapping roles for these pathways in EGF-mediated IEX-I induction. Since IEX-1 has also recently been implicated in regulation of carcinoma growth, selective inhibition of these signaling pathways could be therapeutic targets for treatment of hyperproliferative as well as malignant epidermal disorders.

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Hydrogen Peroxide is a Key Molecule Modulating Arachidonic Acid-Induced Mitogen Activated Kinases Activation and Lamellipodia/Filopodia Formation in Keratinocytes

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Arachidonic acid (AA) is the major precursor of several classes of signaling molecules. Metabolites of AA, or eicosanoids, including prostaglandins, thromboxane, leukotrienes and hydroxyeicosatetraenoic acids, have been implicated as mediators or modulators of a number of physiological functions and pathological conditions in both normal and diseased human skin. We sought to elucidate AA-induced signaling events in keratinocytes. AA activated Rac1 as determined by affinity purification of activated Rac1 through binding to the p21-binding domain of Pak. Rac1 forms part of the NADPH oxidase complex that generates superoxide which is then metabolized to hydrogen peroxide by superoxide dismutase. Rac1 also mediates the activation of c-Jun N-terminal kinase (JNK) and induces lamellipodia formation. Significant amounts of superoxide, nitric oxide and hydrogen peroxide were detected in keratinocytes exposed to AA. We have identified hydrogen peroxide as a mediator of EGF receptor activation, and levels of hydrogen peroxide correlated with EGF receptor tyrosine phosphorylation and active extracellular regulated kinases 1/2 (ERK1/2) in AA treated cells. AA also strongly activated c-Jun N-terminal kinase (JNK) and induced lamellipodia and filopodia formation. Adenoviral overexpression of catalase but not superoxide dismutase completely inhibited cytoskeletal changes and, further, catalase overexpression strongly activated JNK. Cells pretreated with PD153035 also showed increased JNK activity, suggesting an inhibitory effect of hydrogen peroxide-mediated EGF receptor activation on JNK activity. Neither adenoviral catalase nor superoxide dismutase overexpression affected AA-mediated cell death. Our results identify hydrogen peroxide as a key molecule modulating the activation of mitogen activated protein kinases and mediating lamellipodia and filopodia formation.

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Peroxynitrite is a Mediator of Arachidonic Acid-Induced Lipid Peroxidation and JNK Activation

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Arachidonic Acid (AA), a C20 polyunsaturated fatty acid is the precursor for cells to generate cyclooxygenase, lipoxygenase and monooxygenase products and therefore plays a central role in eicosanoid and other lipid synthesis. Metabolites of AA, termed eicosanoids, have been implicated as mediators or modulators of a number of physiological functions and pathological conditions in both normal and diseased human skin. Reactive oxygen species are important regulatory molecules mediating many of the effects triggered by AA. We found that keratinocytes exposed to AA produced both superoxide and nitric oxide in a time dependent manner which strongly correlated with the activation of c-Jun N-terminal kinase (JNK), a stress-induced kinase reported to be activated by end products of lipid peroxidation (LPO). Indeed, AA also induced strong LPO in keratinocytes. Neither nitric oxide nor superoxide alone are strong inducers of LPO. However, peroxynitrite, a potent oxidizing and nitrating species formed in a diffusion-limited reaction between nitric oxide and superoxide, is a well known mediator of LPO. By using antibodies against nitrosylated tyrosine residues and flow cytometry, confocal and Western blotting analysis, we demonstrated that significant amounts of peroxynitrite were generated in keratinocytes exposed to AA. Adenoviral overexpression of superoxide dismutase, by inhibiting LPO, also inhibited JNK activation, whereas catalase overexpression lead to strong activation of JNK, presumably through inhibtion of hydrogen-peroxide-mediated EGF receptor activation. Overexpression of superoxide dismutase or catalase were also examined to determine the role of oxidative stress in mediating AA-induced cell death. We identify peroxynitrite as a mediator of AA-induced JNK activation and LPO.

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A Role for Smad3 in the Senescence Response of Mouse Keratinocytes to Oncogenic Ras

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Alterations in transforming growth factor β (TGF β) signaling and response in mouse keratinocytes have been linked to accelerated premalignant progression *in vivo* and resistance to ras induced senescence *in vitro*. To explore further the possible role of TGFβ signaling as a modifier of skin carcinogenesis, we have compared the tumor and senescence response to activated v-ras^{Ha} of keratinocytes from several inbred mouse strains and the relationship of this response to TGFβ signaling. Following infection with a replication defective retrovirus expressing v-ras^{Ha}, primary Balb/c and C57/BL6 keratinocytes underwent hyperproliferation but then growth arrested and senesced, while FVB/N keratinocytes had a significantly delayed senescence response. When grafted onto nude mice, v-ras^{Ha} transduced Balb/c and C57/BL6 keratinocytes formed primarily benign papillomas while FVB/N keratinocytes rapidly progressed to squamous cell carcinoma. All the three strains had similar growth inhibitory responses to high concentration of exogenous TGFβ1, but following transduction with v-ras^{Ha} Balb/c keratinocytes secreted approximately 2 fold higher levels of total and active TGFβ1 compared to FVB/N. Ras induced senescence of Balb/c and C57/BL6 but not FVB/N keratinocytes was associated with an increase in expression of the cyclin dependent kinase inhibitor $p15^{ink4b}$, a TGFβ responsive gene, suggesting altered TGFβ signaling in FVB/N keratinocytes. Addition of exogenous TGFβ1 and adenovirus mediated overexpression of Smad3 but not Smad2 accelerated senescence in v-ras^{Ha} transduced FVB/N keratinocytes. In addition Smad3–/– keratinocytes had a delayed senescence response associated with poor induction of $p15^{ink4b}$. These results indicate that Smad3 plays an important role in senescence response of mouse keratinocytes to oncogenic ras, and that susceptibility to carcinogenesis in different inbred mouse strains is associated with altered TGFβ signaling.

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Aberrant Association of Wild Type p53 and Mdm-2 at Epidermal Malignant Conversion

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A clonal epidermal cell model of carcinogenesis with nontransformed (291), chemically induced initiated (291.03C), and squamous cell carcinoma (291.03R) derivatives was used to investigate p53 inactivation in multistage carcinogenesis. We previously reported elevated p53 mRNA expression and reduced immunoprecipitable p53 protein in 291.03R without p53 gene mutations. We therefore examined wild type p53 protein defects by immunoblotting of p53-inducible proteins and DNA binding assays of endogenous p53 proteins. The p53 protein was activated in 291 and 291.03C by γ-irradiation: mdm-2 and p21 proteins were induced and p53 and mdm–2 protein interactions were markedly reduced, consistent with stabilization of p53 protein and an intact p53 pathway. In contrast, p53 protein was not induced in 291.03R cells after γ-irradiation and was defective in sequence-specific DNA binding and transactivation of its downstream genes. Although not induced by γ-irradiation due to the p53 defect, mdm-2 protein was constitutively expressed in 291.03R at ~14 fold higher levels than in 291 cells. Mdm-2 abundance alone was not responsible for the lower levels of p53 protein, since p53 protein in 291.03R cells was readily detectable in complex with mdm-2 protein by coimmunoprecipitation. The defect appeared to be in p53 protein activation, since mdm-2 protein from the carcinoma lysate failed to bind activated p53 protein of 291.03C cells. Surprisingly, mdm-2 formed a trimeric complex with p53 and its consensus DNA sequence, suggesting that the p53 protein effector pathway could be defective in part due to mdm-2 protein inhibiting recruitment of p53 N-terminal binding factors required for transcriptional activation. Mdm-2 protein abundance was not due to losses of its regulator protein $p19^{ARF}$, which was expressed and able to associate with mdm-2 protein. Rather, southern analysis revealed mdm-2 gene amplification. These results suggest that defects in wild type p53 protein post-translational modification allowed association of mdm-2 with p53 protein, even when bound to DNA, resulting in enhanced degradation of p53 protein and inhibition of transactivation. Ser15 phosphorylation was inducible in 291.03R, suggesting Thr18 and Ser20 as candidate sites for defects in the upstream pathways of p53 activation. We speculate that the defects in wild type p53 activation foster malignant conversion, genomic instability, and inhibition of p53 protein transcriptional activities.

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Withdrawn

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Profiling Genomic Expression in Disorders of Epidermal Proliferation Identifies Egr-1 as a Potent Regulator of Epidermal Growth

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Epidermal hyperproliferation characterizes a number of skin disorders, including squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and psoriasis. Based on the hypothesis that understanding the underlying gene expression programs responsible for the common and distinct features of these disorders, we performed gene expression profiling on a series of lesional and site matched normal control skin specimens from the same patients (n = 4 for each disorder) and verified these findings with protein expression analysis on a series of 125 patients via immunohistochemical analysis. Initial microarray hybridizations with 12 000 genes were extended to triplicate studies on 1200 genes. Cluster analysis readily differentiated each disorder by pattern grouping, indicating that these disorders display disease-specific patterns of gene expression. The disorders demonstrated the following order of gene expression homogeneity; psoriasis BCC SCC. This is consistent with psoriasis being a reactive disorder of normal tissue, with BCC arising via defined pathways and SCC arising via multiple mechanisms. These disorders displayed commonalties and differences in expression of discrete genes, as also verified by protein expression levels in at least 25 patients for each disorder. PDGF-A was increased in all 3 disorders compared to site matched normal controls, suggesting that the PDGF axis may be a common mediator of epidermal hyperproliferation. Egr-1, a zinc finger transcription regulator, was induced strongly in psoriasis but completely absent in both BCC and SCC, raising the possibility that Egr-1 may help differentiate benign and malignant proliferation. Consistent with this, Egr-1 dramatically inhibited growth of proliferating normal human keratinocytes as well as HPV18 E6/E7 transformed cells and SCC-25 cells. In contrast, a DNA binding domain Egr-1 deletion mutant exerted no growth effects, implicating DNA binding by Egr-1 in epidermal growth suppression. Targeted epidermal expression of Egr-1 in transgenic mice has been achieved, with growth effects currently being analyzed. These data indicate that common disorders of epidermal hyperproliferation can be distinguished on the basis of global gene expression patterns, implicate the PDGF axis as a common mediator and suggest that differential expression of Egr-1 may contribute to differences in biologic behavior of these conditions.

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Integrin $\alpha6\beta4$ Ligation Controls Keratinocyte Morphology and Chemotaxis Through Opposing Stimulation of the Small GTPases, Rac and Rho

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A number of functions of $\alpha6\beta4$ integrin appear to be independent of its attachment to laminin 5. We studied the significance of this to keratinocyte function through point mutation of the $\beta4$ subunit in an identified extracellular attachment domain and its comparison to functional $\beta4$ when transduced by retrovirus into $\beta4$ deficient epidermolysis bullosa keratinocytes (EBK's). Monolayer scratch and Boyden chamber migration assays indicated that functional $\alpha6\beta4$ and laminin 5 are required for induction of chemotaxis through EGF (2 ng per ml). We examined cytoskeletal changes in cells after treatment with EGF. Blank vector (BV) transduced EBK and attachment deficient $\alpha6\beta4$ (AD) EBK's produced only transient (30 min) lamellopodia while wild type $\alpha6\beta4$ (WT) transduced EBK's could sustain lamellopodia formation for at least 4 h. Lamellopodia formation is normally driven through the small GTPase Rac, and retroviral expression of dominant negative (DN) Rac completely inhibited $\alpha6\beta4$ dependant chemotaxis. Unexpectedly, AD EBK's exhibited numerous markers suggesting chronic stimulation of the small GTPase Rho, these included growth factor independent epithelial to mesenchymal transition with prolonged incubation (24 h), cell scattering and increased stress fibers and focal adhesions containing $\alpha3\beta1$ integrin. Transduction of AD EBK's with DN Rho completely reversed the mesenchymal phenotype. Furthermore, DN Rac transduced WT EBK's exhibited profound mesenchymal transformation while BV transduced DN Rac EB cells remained epithelial. Since Rac activation impairs Rho in epithelial cells we conclude that $\alpha6\beta4$ integrin controls an opposing balance of attachment dependant Rac activation and attachment independent Rho activation. Control of this balance is essential for EGF induced chemotaxis, while an imbalance in these opposing forces may explain many of the alternative functions of $\alpha6\beta4$ in transformed cells.

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Loss of Expression of p16INK4a and p14ARF Genes Via Deletion and Promoter Hypermethylation in Human Non Melanoma Skin Cancers

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The INK-ARF locus localized on human chromosome 9p21 encodes two alternatives reading frame proteins (p16INK4a and p14ARF) known to function as tumor suppressors via the retinoblastoma or the p53 pathway. Inactivation of p16INK4a can lead to dysregulation of these two pathways. Although mutations in the p53 gene are well documented in human nonmelanoma skin cancers (NMSC), the role of p16INK4a and p14ARF in the development of human NMSC is not well studied. We therefore analyzed 40 NMSC (21 primary human squamous cell carcinomas, 17 basal cell carcinomas and 2 actinic keratoses) for alterations in p16INK4a and p14ARF genes by PCR. The results indicated that about 85% of the tumors had deletions in exon 2 of the p16INK4a gene. In addition, methylation-specific PCR experiments revealed that about 45% of the tumors had hypermethylation in the p16INK4a and p14ARF promoter regions. Immunohistochemical analysis revealed loss of expression of p16 and p14 proteins in 97% of NMSC. As expected, about 80% of human NMSC analyzed contained UV signature mutations in the p53 gene and almost all of them were strongly positive for p53 immunostaining. Based on these data, we conclude that in addition to mutations in the p53 gene, loss of expression of p16INK4a and p14ARF genes via deletion and promoter hypermethylation also plays an important role in the pathogenesis of human NMSC.

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Keratinocyte Growth Inhibition by High Doses of Epidermal Growth Factor is Mediated Through Autoinduction of Transforming Growth Factor β: A Negative Feedback Mechanism of Keratinocyte Growth

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The epidermal growth factor receptor (EGFR) ligand-EGFR signal is a major signaling pathway that regulates keratinocyte growth in an autocrine manner. However, it is well known that high doses of EGFR ligands inhibit keratinocyte growth. Recently, STAT1-dependent $p21^{Waf1/Cip1}$ induction was reported as a mechanism of EGF-dependent cell growth arrest in an A431 squamous carcinoma cell line. However, transfection of dominant negative STAT1 adenovirus vector did not block EGF-induced growth inhibition in normal keratinocytes. Since transforming growth factor β (TGFβ) is a potent inhibitor of keratinocyte proliferation, we hypothesized that TGFβ contributed to EGF-mediated keratinocyte growth inhibition. First, we used p3TP-lux, which contains three tandem TGFβ-smad signaling responsive elements, to examine whether TGFβ acts in an autocrine manner in normal human keratinocytes. 100 ng per ml EGF up-regulated p3TP-lux luciferase activity 8-fold compared to no stimulation. This EGF-dependent induction of p3TP-lux luciferase activity was disrupted by transfection of the dominant negative form of type 1 TGFβ receptor (ALK5) adenovirus vector, which indicated that EGF-induced TGFβ acts in an autocrine manner in keratinocytes. Consequently, 10–100 ng per ml EGF induced TGFβ1 mRNA in a dose- and time-dependent manner, and about 40 pg per ml per 10^6 cell of TGFβ1 was detected in culture medium incubated with 100 ng per ml EGF using an ELISA method, but 0.1–1 ng per ml EGF did not induce TGFβ1 production. Moreover, transfection of the dominant negative form of ALK5 adenovirus vector completely blocked the growth inhibition induced by 100 ng per ml EGF in normal keratinocytes, which indicated that EGF-induced TGFβ mainly inhibits keratinocyte proliferation. These data demonstrate that an autocrine TGFβ-ALK5 pathway is a negative feedback mechanism for EGFR-dependent normal keratinocyte growth.

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Collagen-Activated Keratinocyte Collagenase-1 Expression Requires ERK 1/2 MAP Kinase Signaling

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Following injury, keratinocytes switch from a gene expression program that promotes differentiation to one that supports migration. A hallmark of the activated keratinocyte is the precise and invariant expression of collagenase-1 (MMP-1) at the leading edge migration in wounds with a disrupted basement membrane. Previously, we have shown that contact with type I collagen mediates keratinocyte MMP-1 expression via a2β1 integrin signaling. Recent evidence from others has shown that cell type-specific signaling cascades regulate induction of collagenase-3 (p38 MAPK) and gelatinase B (jun kinase) in fibroblasts and endothelial cells, respectively. We assessed if the intracellular signaling mechanism(s) mediating collagen induction of keratinocyte MMP-1 is controlled by similar pathways. Within 10 min following collagen contact, extracellular signal-regulated kinase 1,2 (ERK 1/2) and p38 MAPK were markedly phosphorylated, whereas JNK phosphorylation remained at basal levels. Blocking the ERK 1/2 pathway with MEK 1/2 inhibitors PD98059 or UO126 dramatically inhibited collagen-stimulated MMP-1 protein and mRNA and transcription from a 2.2-kb MMP-1 promoter-luciferase construct. In contrast, treatment with the p38 inhibitor SB203580 or the JNK inhibitor curcumin had no effect on MMP-1 production. Because MMP-1 activity is required for keratinocyte migration across type I collagen, we assessed the effects of these signaling inhibitors on cell motility. Migration of keratinocytes across collagen was inhibited by treatment with PD98059 and UO126, but relatively unaffected by SB203580 or curcumin. These results demonstrate that ERK 1/2 MAPK signaling is required for collagen-mediated induction of keratinocyte MMP-1 and provide further evidence that cell type-specific signaling cascades regulate MMP expression by a variety of stimuli.

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Reciprocal Changes in the Expressions of Nicotinic Acetylcholine Receptors, and the Cell Cycle and Differentiation Genes can Mediate Pathobiologic Effects of Tobacco Products on Keratinocytes

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Smoking and smokeless tobacco cause morbidity originating from the epithelium lining skin and upper digestive tract. Keratinocytes (KC) express nicotinic acetylcholine receptors (nAChRs) that bind nicotine (Nic). We studied the mechanism of receptor-mediated toxicity of tobacco products on KC. Preincubation of normal human oral KC with Nic altered ligand binding kinetics of their nAChRs, suggesting structural changes of nAChRs. This hypothesis was confirmed by finding transcriptional and translational changes in the exposed KC. From 1.5 to 2.9 fold increase of mRNA and protein levels of α3, α5, α7, β2, and β4 nAChR subunits could be detected by semiquantitative RT-PCR and immunoblotting. Exposure of KC to Nic also changed their mRNA and protein levels of Ki-67, PCNA, p21, cyclin-D1, p53, filaggrin, loricrin and cytokeratin 10. These alterations could be abolished in the presence of the nicotinic antagonist mecamylamine, indicating that Nic-induced changes of the expression of both the nAChR and the cell cycle/differenatiation genes resulted from a pharmacologic stimulation of nAChRs. To establish relevance of these findings to the pathobiologic effects of tobacco products *in vivo*, we studied the above parameters in the oral tissue of rats and mice after their exposures for 3 weeks to the environmental cigarette smoke or drinking water containing equivalent concentrations of Nic. The changes of nAChRs and cell cycle/differentiation were similar to those found *in vitro*, and semiquantitative immunofluorescence of tissue specimens validated these findings. Thus, some pathobiologic effects of tobacco products in the skin and oral tissues may stem from Nic-induced alterations of structure/function of nAChRs that alter the physiologic regulation of the cell cycle by the cytotransmitter acetylcholine.

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Rapid and Sustained Induction of Smad7 in the Epidermis and Hair Follicles of Transgenic Mice Conditionally Expressing Active TGFβ1

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We have previously established a conditional transgenic expression system for active TGFβ1 using the tetracycline regulated transactivators tTA and rTA targeted to the epidermis with the bovine keratin 5 promoter, and the constitutively active mutant of TGFβ1 linked to the tetO binding site for the tTA and rTA. Overexpression of TGFβ1 in the epidermis of adult mice causes a reversible alopecia associated with hyperplasia and increased apoptosis. To identify primary targets of TGFβ1 which could contribute to this phenotype, we have examined expression of Smad7, an inhibitory Smad which can block TGFβ mediated signal transduction and is itself induced by TGFβ *in vitro*. In the alopecic skin of bigenic mice overexpressing TGFβ1, Smad7 protein was highly expressed compared to the single transgenic and uninduced bigenic mice. Indirect immunofluorecsence showed that Smad7 expression was cytoplasmic and predominantly localized to the suprabasal layer of the epidermis and inner root sheath of hair follicles. In K5/rTA double transgenic mice, the induction of Smad7 mRNA and protein occurred rapidly and closely paralleled induction of TGFβ1 mRNA following addition of doxycycline. A similar pattern of induction occurred in K5/tTA mice removed from doxycycline. In primary Balb/c keratinocytes infected with a Smad7 adenovirus, both the TGFβ1 mediated induction of gene expression from the 3TP-lux reporter plasmid and TGFβ1-induced growth inhibition were blocked. To further examine the effects of Smad7 on TGFβ1-mediated gene expression, cDNA microarray expression profiles were compared between TGFβ1 treated control and Smad7 adenovirus infected primary keratinocytes. Interestingly, Smad7 blocked most TGFβ1 induced genes including extracellular matrix proteins, and by itself Smad7 also altered gene expression in primary keratinocytes. These results show that Smad7 is a primary target of TGFβ signaling *in vivo* and suggest its prolonged expression may significantly alter the response of keratinocytes to TGFβ1, and contribute to the development of alopecia.

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MAP Kinase Cascade Regualtion of Human Papillomavirus Transcription in Keratinocytes – a Differential Role for Erk1 and Erk2

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Human papillomavirus (HPV) is an important disease-causing entity in the epidermis, cervix, and oral cavity. The HPV upstream regulatory region (URR) promoter drives transcription of the viral oncoproteins, E6 and E7, in a differentiation-dependent manner; however, little is known regarding the signal transduction cascades that regulate URR activity. To identify these signals, we use a reporter construct encoding the human papillomavirus type 16 (HPV16) upstream regulatory region (URR) that drives transcription in normal keratinocytes. TAM67, a dominant-negative mutant of c-jun that inactivates activator protein 1 (AP1) transcription factors, reduces URR activity. Likewise, GADD153, a dominant-negative CCAAT/enhancer binding protein (C/EBP) isoform, reduces expression. These results suggest that the AP1 and C/EBP transcription factor binding sites present in the URR are important for expression. We next evaluated the role of various signal transduction kinases. Constitutively active Ras markedly increases promoter activity, while dominant-negative Ras inhibits expression. Dominant-negative MEKK1 also markedly reduces URR activity. This Ras- and MEKK1-associated regulation appears to target the ERK family of mitogen activated protein kinases (MAPK). Studies using dominant-negative ERK isoforms, suggest that ERK1 is a strong suppressor and ERK2 a strong activator of URR activity. Additional studies show that the URR DNA element that mediates this regulation is localized in a small region proximal to the transcription start site that contains AP1 and C/EBP factor binding sites. These results suggest that a Ras, MEKK1, MEK1, ERK1/2 mitogen-activated protein kinase cascade that targets AP1 and C/EBP transcription factors regulates HPV promoter activity in keratinocytes via a unique mechanism that involves differential activation of ERK1 and ERK2.

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Epithelial Interactions with Basement Membrane Control Early Neoplastic Progression in Tissue Models of Premalignant Disease

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Perturbations of epithelial–stromal interactions are features of advanced malignancy but their role in the initiation and progression of premalignant disease is unclear. We have used novel *in vitro* and *in vivo* human tissue models for premalignancy of stratified epithelium to dissect the contribution of basement membrane components to early neoplastic progression in this tissue. This was accomplished by following the fate and behavior of intraepithelial tumor cells which were genetically marked with the gene for β-galactosidase and mixed with normal human keratinocytes (NHK) at a 4:1 ratio (NHK:tumor cells), to generate skin-like, organotypic cultures which were assembled in the presence or absence of basement membrane. We found that low-grade (HaCaT-II-4), intraepithelial tumor cells were retained in a basal position *in vitro* and underwent intraepithelial expansion after transplantation to nude mice to generate a dysplastic epithelium when grown in the presence of basement membrane. In contrast, these tumor cells were sorted to the suprabasal compartment and did not persist after grafting when grown without basement membrane. To determine if a particular basement membrane component was associated with neoplastic progression, 4:1 mixtures were seeded on polycarbonate membranes coated with purified basement membrane components (Type IV collagen, fibronectin, Type I collagen or fibronectin-type I collagen) which were placed on a contracted collagen gel containing dermal fibroblasts. Only Type IV collagen was found to be permissive for the intraepithelial expansion of II-4 cells, suggesting that this component directed the adhesion and persistence of II-4 cells. In contrast, when a high-grade tumor cell line (SCC13) was mixed with normal cells, it was found that these cells did not require contact with basement membrane components to undergo expansion and invasion. Our findings demonstrate that intraepithelial tumor cells at an early stage of transformation are sensitive to microenvironmental control by basement membrane. This may contribute to the regulation of early cancer progression in human stratified squamous epithelium.

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Metalloproteinase Inhibition Blocks Invasion of Squamous Cell Carcinoma Cells

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Increased metalloproteinase activity correlates with increased invasiveness of most tumor cells. Matrilysin, MMP7, is the only metalloproteinase expressed by epithelial cells, and in skin, MMP7 expression is elevated in aggressive forms of squamous cell carcinoma (SCC). TIMP-3, a metalloproteinase inhibitor, shows the same expression pattern in skin and SCC as MMP-7. Therefore, the effects of metalloproteinase activity on invasiveness of SCC cells was examined. Four cell lines were tested in a matrigel invasion assay. FaDu and A431 cells were highly invasive, but SiHa and HaCat did not invade matrigel. BB-94 (Batimastat), a synthetic metalloproteinase inhibitor, blocked matrigel invasion in both FaDu and A431 cells, but stimulated invasiveness in HaCat cells. Expression of mRNA for MMP7 and TIMP-3 was examined by semiquantitative RT-PCR. The ratio of MMP7/TIMP-3 was highest in the most invasive cells, FaDu, and lowest in the noninvasive HaCat cells, indicating that TIMP-3 regulates MMP7 activity. Immunoblotting for pro-MMP7 and active MMP7 demonstrated that in the noninvasive HaCat cells, inactive pro-MMP7 predominated, whereas in the highly invasive FaDu cells, active MMP7 was predominant. These results suggest that MMP7 plays an important role in SCC invasiveness and that inhibitors of metalloproteinases may prove therapeutic in the treatment of squamous cell carcinoma.

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Fas/FasL Pathway Involved in Arsenic Induced p53-Dependent Apoptosis in Cultured Keratinocytes

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Epidermiological studies demonstrated that long-term exposure to arsenic induces arsenical skin cancers, including Bowen's disease. Histologically, it shows proliferating and apoptotic characteristics at Bowen's disease lesion. To investigate how arsenic induces cellular apoptotic death, we exposed cultured human foreskin keratinocytes to different concentrations of sodium arsenite for 48 h, the expression of apoptotic proteins and receptors were detected by Western blotting and flow cytometry. Arsenic enhanced keratinocytes proliferation at low concentrations, however, apoptosis was induced at high arsenic concentrations (¡Ù5 μM). Arsenic enhanced p53 expression and arrested keratinocytes cell cycle at G0/G1 phase. The level of apoptotic protein Bax was increased in a Bcl-2 down regulated manner when keratinocytes were exposed to arsenic. Arsenic could also affect expression of transcriptional factors. Arsenic enhanced not only the quantity of c-Jun/c-Fos proteins in keratinocyte, the phosphorylation of c-Jun was also enhanced, which indicated the activation of AP-1 signals. However, the cellular level of NF-κB was down regulated while the expression of IκB-α increased. The results of arsenic induced AP-1 activation and NF-κB decrease indicate a tendency of apoptotic death at the signal transduction level. The expression of several apoptosis related receptors were then detected, we found cell membrane Fas and Fas ligand (FasL) increased through arsenic treatment. Our results suggest that Fas/FasL pathway is involved in arsenic induced p53-dependent apoptosis in cultured keratinocytes.

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Involvement of Retinoblastoma (Rb) and E2F Transcription Factors During Epigallocatechin-3-Gallate-Mediated Cell Cycle Dysregulation and Apoptosis of Human Epidermoid Carcinoma A431 Cells

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Epidemiological, *in vitro* cell culture and *in vivo* animal studies have shown that green tea or its constituent polyphenols, particularly its major polyphenol epigallocatechin-3-gallate (EGCG) may protect against cutaneous inflammatory responses and cancer skin cancer. Consistent with this notion, many cosmetic- and skin care- products supplemented with green tea are available at present. Studies from this laboratory have shown that EGCG causes a G0/G1-phase cell cycle arrest and apoptosis of human epidermoid carcinoma (A431) cells. We also showed the involvement of nuclear factor κB (NF-κB) pathway and cyclin kinase inhibitor (cki)-cyclin-cyclin dependent kinase (cdk) machinery during cell cycle deregulatory/apoptotic response of EGCG. A complete understanding of the mechanism of antiproliferative effects of green tea polyphenols is important. The proteins of the retinoblastoma family pRb, p107 and p130, collectively referred to as "pocket proteins", in association with E2F family transcription factors, have been shown to regulate the G1-S transition in the cell cycle. The activity of the Rb proteins is regulated by cyclin-cdk network-mediated phosphorylation. Here, we provide evidence for the involvement of pRb-E2F/DP machinery as an important contributor of EGCG-mediated cell cycle dysregulation and apoptosis. Immunoblot analysis demonstrated that EGCG treatment of A431 cells resulted in a dose- as well as time- dependent decrease in the hyper-phosphorylated form of pRb with a relative increase in hypo-phosphorylated pRb. Immunoblot analysis also revealed that EGCG-caused decrease in phosphorylation of pRb occurs at serine-780. EGCG treatment of A431 cells was also found to result in dose- and time- dependent inhibition of other Rb family proteins viz. p107 and p130. These responses were accompanied with down-modulation in the protein expression of all five E2F (1 through 5) family transcription factors, and their heterodimeric partners DP1 and DP2. These results suggest that EGCG causes a down regulation of "pocket proteins" that, in turn, compromises with the availability of free E2F. We suggest that these events result in a stoppage of the cell cycle progression at G1-S transition thereby leading to a G0/G1 phase arrest and a subsequent apoptotic cell death.

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The Role of Caspases in Execution of Apoptosis in Human Epidermoid Carcinoma A431 Cells by Green Tea Polyphenol (–) Epigallocatechin-3-Gallate

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Our studies have established that green tea possesses anti-inflammatory and anticarcinogenic effects in the skin and that epigallocatechin-3-gallate (EGCG); the major polyphenol present therein is responsible for most of its biological activity. Earlier we have shown the involvement of apoptosis in the antiproliferative effects of EGCG. Because EGCG was shown to exhibit preferential apoptosis in cancer cells as compared to normal cells, a complete understanding of sequence of events by which EGCG mediates apoptosis is important. Since caspase activation is regarded as a triggering event in execution of apoptosis, we assessed the activation of caspases during EGCG-mediated apoptosis of human epidermoid carcinoma A431 cells. Treatment of cells with EGCG resulted in dose- and time- dependent apoptosis as shown by the formation of DNA ladder, PARP cleavage and by TUNEL assay. Further, EGCG treatment (20 μg per ml) of cells resulted in a significant activation of caspases as shown by an increase in DEVDase activity, assessed by measuring the release of 7-amino-4-trifluoromethyl coumarin (AFC) from N-acetyl-Asp-Glu-Val-Asp (DVED)-AFC substrate. Immunoblot analysis demonstrated a dose- as well as time-dependent up-regulation in the protein expression of caspase-3, –8 and –9 following EGCG treatment. Pretreatment of cells with the general caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK) prevented EGCG-mediated apoptosis as assessed by (a) TUNEL positive cells, (b) PARP cleavage, and (c) fluorescence microscopy. Similar apoptosis inhibitory effects although of the lower magnitude, were observed with specific caspase-3 inhibitor, Z-DVED-FMK. Treatment of A431 cells with Z-VAD-FMK also resulted in inhibition of the protein expression of caspase –3, –8 and –9. Since bax/bcl2 has been shown to be involved in caspase activation and apoptosis, we next assessed the effect of EGCG on the levels of bax and bcl2 proteins. As shown by immunoblot analysis, EGCG treatment was found to result in a dose- and time-dependent increase in the levels of bax without affecting bcl2, thereby shifting the ratio in favor of apoptosis. Taken together, based on our data we suggest that EGCG modulates bax/bcl2 ratio followed by caspase activation, which in turn triggers apoptotic death of cancer cells.

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Paclitaxel-Induced Apoptosis is Dependent on Activation of Caspase-8 and 3 with a Preceding Activation of Survival Modulator Akt in Cultured Human Keratinocytes

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Paclitaxel is a natural antimicrotubule agent shown to possess antitumor activity. It is currently used as a chemotherapeutic agent in the treatment of ovarian and breast carcinomas. We investigated the molecular effects of paclitaxel on cultured human keratinocyte cells (HaCaT), assessing the induction of both the apoptotic pathway and the cell survival signals. Results from MTT dye assay indicated that paclitaxel reduced keratinocyte growth in a 3-day bioassay with an effective $ED_{50}$of 6–600 ng per ml. A large variation in ED_{50}can be attributed to the asynchronous population of cells. Western Blot analysis showed that paclitaxel treatment did not significantly affect phosphorylation of bcl-2 in cultured keratinocytes. Results did indicate that within 6 h of paclitaxel exposure there was an increase in both Caspase 8 and 3 activities, indicating the activation of apoptotic pathways. Paclitaxel treatment also induced phosphorylation of Akt (Ser473 and Ser308) in a time dependent manner. AKT phosphorylation was detectable at 30 min post paclitaxel treatment (600 ng per ml), peaked at about 1 h and remained elevated up to 2 h. These results indicate that paclitaxel activates PI3-kinase/AKT cell survival pathway while inducing apoptotic machinery in cultured human keratinocytes. Collectively, our data indicate that (a) paclitaxel-induced apoptosis is independent of the bcl-2 phosphorylation, (b) the blockade of PI 3-kinase/AKT pathway may help potentiate the apoptotic effects of paclitaxel and (c) the apoptotic signaling pathways are initiated with the activation of caspase-8 and 3 activities.

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p53 Stabilizing Compound, CP31398, Induces Apoptosis by Activating Caspases

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p53 is considered the guardian of the genome. Wild-type (wt) p53 has a number of biological functions including cell cycle arrest, DNA repair and apoptosis. In a recent study by Foster and colleagues, the pharmacological compound CP31398 was found to stabilize wt p53, enabling it to activate transcription and slow tumor growth in mice. CP31398 is also able to revert mutant p53 back to wild-type conformation. We hypothesize that CP31398 induces apoptosis by stabilizing p53. We have found that CP31398 induces cell death in the p53 wt melanoma cell lines MMRU and MMAN, while the mutant p53 melanoma cell lines MEWO and SK110, were more resistant. To further investigate the mechanisms of CP31398-induced cell death, we used two colon carcinoma cell lines, HCT116+/+ which contains wt p53 and HCT116−/− which lacks the p53 gene. We demonstrated that CP31398 kills cancerous cells by triggering apoptosis in a dose- and p53-dependent manner. Furthermore, we found that CP31398 induces cleavage of caspase-9 and −8, suggesting that it activates the mitochondrial- or death receptor-mediated apoptotic pathways, respectively. In addition, p21waf1 is up-regulated after CP31398 treatment, implying that it may be involved in cell cycle growth arrest. Taken together, our results indicate that CP31398 induced cell death is p53-dependent. Elucidating the mechanism by which CP31398 induces cell death may allow it to be used as anticancer treatment for different forms of cancer.

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The Epidermal Platelet Activating Factor Receptor Protects Squamous Cell Carcinoma Cells Against Chemotherapy-Induced Apoptosis

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The mechanism of action of many chemotherapeutic agents involves the induction of apoptosis on malignant target cells. Recent studies suggest that cytokines produced by carcinomas such as interleukin-1 can protect against apoptosis induced by various agents. Though the lipid mediator platelet-activating factor (1-alkyl-2-acetyl-glycerophosphocholine; PAF) is synthesized by keratinocytes and carcinoma cell lines in response to diverse stimuli, and these cell types express PAF receptors (PAF-R), it is not known whether the PAF system can affect chemotherapeutic agent-induced toxicity. Using a novel model system created by retroviral-mediated transduction of the PAF receptor-negative human epidermal cell line KB with the human PAF-R, we tested whether the PAF-R could modulate toxicity induced by etoposide and mitomycin C. Treatment of control or PAF-R-expressing KB cells with etoposide and mitomycin C resulted in a dose-dependent increase in caspase 3, indicating that these agents induced apoptosis in these cell types. Preincubation of KB cells with the PAF-R agonist CPAF resulted in decreased apoptosis in response to etoposide and mitomycin C only in PAF-R-expressing cells. Treatment of KB cells with two separate PAF-R antagonists inhibited the protective effect of CPAF, indicating CPAF exerted its protective effects via activation of the epidermal PAF-R. These studies demonstrate that the PAF system can protect PAF-R-positive carcinoma cells against chemotherapeutic agents. Inasmuch as PAF would be expected to be generated during an inflammatory response, these findings might provide a clinically relevant mechanism by which carcinoma cells may be protected against chemotherapeutic agents.

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Curcumin Decreases Spontaneous Proliferation and Induces Apoptosis in Squamous Cell Carcinoma Cell Lines *In Vitro*

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Curcumin (diferuloylmathane), is the major chemical constituent of turmeric, a spice commonly used in many Asian cultures. Curcumin has potent antioxidant effects, and has been reported to significantly inhibit experimentally induced tumorigenesis on mouse skin. The exact mechanism of its antioxidant and chemopreventive activity is not known. However, curcumin inhibits arachidonic acid-induced inflammation in mouse skin and triggers apoptosis in several tumor cell lines. We tested the effects of curcumin on the spontaneous proliferation and viability of HaCaT and A-431, two human keratinocyte-derived carcinoma cell lines. Treatment with curcumin inhibited the spontaneous proliferation of both carcinoma cell lines using a tetrazolium-based (MTT) cell proliferation assay in a dose-dependent manner. Curcumin treatment decreased cell viability and triggered caspase 3 enzymatic activity, suggesting that this natural product induces apoptosis in squamous cell carcinoma cell lines. These studies provide preliminary data suggesting that curcumin could have therapeutic potential in the treatment of human cutaneous squamous cell carcinomas.

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Increased Resistance to Apoptosis in FGFR3 Mutant Keratinocytes is Not Mediated by STAT3 Phosphorylation

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Mutations that result in constitutive activation of fibroblast growth factor receptor 3 (FGFR3) cause several different skeletal dysplasia and craniosynostosis syndromes, some of which are associated with acanthosis nigricans. Several investigators have shown that these FGFR3 mutations lead to phoshorylation of STAT1, growth arrest and increased susceptibility to apoptosis in chondrocytes. In addition to skeletal dysplasias, activating FGFR3 mutations have been reported in several different malignancies including multiple myeloma, bladder and cervical cancers. In multiple myeloma, FGFR3 mutations result in phosphorylation of STAT3, increased exression of Bcl-XL and increased cell survival. We have reported previously that keratinocytes from patients with acanthosis nigricans due to FGFR3 K650M and A391E mutations exhibit increasesd expression of Bcl-2 and resistance to apoptotic triggers. In order to investigate whether increased survival in keratinocytes with FGFR3 mutations is mediated by STAT3 we evaluated pSTAT3 levels by immunoblots in control and FGFR3 mutant keratinocytes (K650M) under actively growing conditions and after several different apoptotic triggers. Control and mutant keratinocytes expressed very little pSTAT3 under all conditions tested. We conclude that increased resistance to apoptosis in FGFR3 mutant keratinocytes is not mediated by activation of STAT3 and must involve other signaling pathways. These results further demostrate that cellular outcomes due to FGFR3 signaling is highly tissue specific.

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Signaling for Growth or Apoptosis Through the 75 kDa Neurotrophin Receptor

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p75, nerve growth factor (NGF) coreceptor, is a member of the tumor necrosis factor (TNF) receptor superfamily and is expressed by melanocytes (Mc). We have shown that NGF a keratinocyte cytokine, activates p75 on Mc to induce proliferation at 5 nM but death at 60 nM. TNF-α receptors are present as trimeric complexes and as monomers, but signal apoptosis only when ligand binds receptor trimer. To determine p75 organization and its bearing on signaling, Mc were treated with a cross linker. By Western blotting p75 exists as a preformed trimer and as a monomer. To explore NGF-mediated p75 signaling, Mc and p75 expressing 3T3 cells were provided 5 or 60 nM NGF. Within minutes, as shown by Fluo-3-Am NGF increased intracellular Ca2+. However, 5 nM NGF increased Ca2+ in the cytosol but 60 nM elevated Ca2+ also in nuclei, consistent with Ca2+ requirement for apoptotic endonuclease activity. Pretreatment with a cyclic peptide CATDIKGKEC (cycP) that blocks NGF binding to p75 abrogated this response. Moreover, only 60 nM NGF induced apoptosis associated activation of c-Jun amino terminal kinase (JNK) as measured by phosphorylation of its substrate, and this response was abolished by cycP. Further, Bax mRNA and protein were strongly induced within 24 h of 60 nM NGF and anti-BAD as well as anti-BID antibodies, showed substantial induction of these proteins that act upstream of Bax, consistent with caspase 8 activation. Sustained c-myc protein induction accompanied this response. Finally, both TUNEL analysis and cytoplasmic DNA-histone levels proved that these signaling lead to apoptosis that was abrogated by cycP. In contrast, 5 nM NGF led to brief induction of c-myc and cyclin D that was abrogated by cycP. We show that different NGF concentrations induce opposing events in Mc and speculate that high NGF doses activate p75 trimers leading to apoptosis, whereas low NGF levels activate p75 monomers to induce proliferation. NGF's differential effects may explain irregular Mc distribution in sun damaged skin.

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The Epidermal Platelet Activating Factor Receptor Protects Keratinocytes from TNF-α and TRAIL-Induced Apoptosis Through a Nuclear factor κB-Dependent Process

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A number of chemical mediators can induce keratinocytes and keratinocyte-derived carcinomas to undergo apoptosis, or programmed cell death. Recent evidence suggests exposure to cytokines, such as IL-1B, results in an antiapoptotic effect, protecting keratinocytes from pro-apoptotic stimuli. The platelet activating factor receptor (PAF-R), present on keratinocytes, is a G protein-coupled receptor activated by the pro-inflammatory lipid mediator platelet activating factor (PAF). Since PAF is produced by keratinocytes in response to diverse stimuli, and the keratinocyte PAF-R is involved in numerous cell functions, we utilized a model system created by retroviral-mediated transduction of the PAF-R into the PAF-R-negative human epidermal cell line (KB) to assess the effect of PAF on tumor necrosis factor-α (TNF-α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Exposure to TNF-α or TRAIL induced apoptosis in PAFR-negative (KBM) and PAFR-positive (KBP) cells in a dose and time dependent-manner. Pretreatment with the PAF-R agonist, CPAF, protected KBP, but not KBM cells from TNF-α and TRAIL-induced apoptosis, whereas exposure to IL-1B protected both KBP and KBM cells from these aptogens. Pre-incubation with PAF-R antagonists blocked the antiapoptotic activity of CPAF, but not IL-1B, in KBP cells. We next sought to identify the cellular mechanisms mediating the antiapoptotic activity of PAF. Exposure to CPAF or IL-1B induced a rapid (within 15 mins) degradation of IKB, the inhibitor of nuclear factor-κB (NF-κB), resulting in a maximal increase in NF-κB within 1 h. Retroviral-mediated transduction of KBP cells with a super-repressor IKB, blocked the CPAF-induced increase in NF-κB and abolished the antiapoptotic effects of CPAF. These studies indicate that activation of the PAF-R protects KB cells against TNF-α and TRAIL-induced apoptosis through a NF-κB dependent pathway. Furthermore, this study is the first to demonstrate that activation of a G protein-coupled receptor can attenuate apoptosis induced by members of the TNF-α receptor super-family. Since PAF is produced by epidermal cells in response to numerous stressors, we hypothesize that this mediator exerts a protective effect against pro-apoptotic stimuli generated by immune cells

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Inhibitory Effects of Selected Chinese Herbs on UV- or TNF-α-Induced Activation of p38 MAP Kinase in Cultured Human Keratinocytes

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Thus far accumulated evidence have established that UV-induced skin damage is largely due to two major cellular/molecular events including (1) overexpression of MMPs that destroy skin connective tissues through MAP kinase pathway and AP-1 activation and/or (2) overexpression and release of cytokines through NF-κB pathway. Reagents from synthesized chemical library and natural resources have been extensively searched to prevent UV- or cytokine-induced activation of MAP kinase and NF-κB pathway. Using cultured human keratinocytes we tested a group of selected Chinese herbs that have been known in long history of traditional Chinese medicine to be effective in anti-inflammation or in beautifying human skin, to investigate the uncovered molecular mechanisms. Among those we found that extracts from TQDHY (*Gynura bicolor*DC), YXC (*Houttuynia cordata*Thunb), and ZHDD (*Viola yedloensis*Mak) dramatically inhibited TNF-α-induced phosphorylation of p38, to less extent inhibited UV-induced p38 phosphorylation, but had no effect on TNF-α- or UV-induced JNK phosphorylation. These data suggest that those herb extracts may not reversibly act merely as antioxidants. Both water- and DMSO-soluble fractions from TQDHY inhibited TNF-α-induced p38 phosphorylation in a time and dose dependent manner. Surprisingly, all herb extracts tested had no effects on TNF-α-induced IκB degradation, suggesting that those herbs do not interfere with NF-κB pathway. We contend from our data that those selected Chinese herbs target p38 MAP kinase pathway and could be applied to prevent UV-induced skin photoaging and skin inflammation.

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Cyclosporin A Induces Secretion of Cyclophilin B Via the Constitutive Pathway in Human Keratinocytes

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Cyclosporin A (CsA) is effective treatment for inflammatory skin diseases. Its mechanism of action in T-cells is well characterized, although the effects of CsA in nonimmune tissue and skin are less well understood. Cyclophilin B (CypB) is one member of the ubiquitous family of proteins known as cyclophilins which are defined by their ability to bind CsA and to catalyze the isomerisation of peptidyl-prolyl bonds. CypB is a 21-kDa endoplasmic reticulum resident protein, which in T-cells contributes to mediating CsA effects and is secreted in response to CsA. However, the physiological role of CypB remains to be fully defined. The aim of this study was to investigate whether CypB is expressed in skin, if CsA modulates the intracellular location of CypB, and if CsA promotes secretion of CypB in keratinocytes. Immunofluorescence staining showed expression of CypB by suprabasal keratinocytes and Western blotting confirmed that CypB is expressed by keratinocytes. Cultured human keratinocytes were treated with CsA (1 μM) for 15 min, 2 h or 18 h and then either (1) fixed and double labeled with an anti-CypB antibody and BODIPY-ceramide, a probe for the golgi apparatus (n = 3), or (2) culture medium was collected, concentrated and analysed using Western blotting (n = 3). In untreated cells, punctate cytoplasmic staining and strong nuclear staining of CypB was observed. After treatment with CsA, cytoplasmic CypB redistributed to the golgi apparatus and also was found to localise at the plasma membrane. Western analysis showed that CypB accumulated in the medium in response to CsA in a time dependent manner, which could be completely inhibited by pretreatment with brefeldin A, a potent inhibitor of constitutive secretion. These data shows that keratinocytes constitutively secrete CypB in response to treatment with CsA. This suggests that CypB displays similar functions in keratinocytes as those observed in T cells, and therefore may play a role in mediating the action of CsA in inflammatory skin disease.

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Adherens Junction Mediated Signaling in Human Keratinocytes: Support for the Hypothesis

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β-catenin and plakoglobin function in adherens junction mediated cell-cell adhesion and in Wnt mediated signal transduction. We have previously shown that tyrosine phosphorylation of β-catenin and plakoglobin reversibly regulates their association with E-cadherin and α-catenin in human keratinocyte adherens junctions. In Wnt mediated signal transduction, binding of extracellular Wnt to Frizzled family receptors results in formation of transcriptional regulatory complexes between β-catenin and TCF/LEF family members. We hypothesize that the cadherin family of cell-cell adhesion proteins transduce signals from the extracellular to the intracellular compartment and that β-catenin and plakoglobin are integral components of cadherin signaling cascades. By translocation from cell-cell junctions to the nucleus, β-catenin and plakoglobin could directly link the "sensing" of altered cell-cell adhesion at the cell surface to transcription in the nucleus. To investigate this hypothesis, normal human keratinocytes in tissue culture were treated with the tyrosine phosphatase inhibitor peroxovanadate and the distribution of plakoglobin examined by (i) immunofluorescent confocal microscopy and (ii) subcellular fractionation and Western blot analysis. The ability of plakoglobin to bind to TCF/LEF family members was investigated by coimmunoprecipitation and gel shift assays. After treatment with peroxovanadate, keratinocytes displayed (i) increased nuclear localization of plakoglobin, (ii) tyrosine phosphorylation of nuclear plakoglobin, and (iii) increased complex formation between TCF/LEF family members and plakoglobin in nuclear fractions. Our results indicate that plakoglobin, released from adherens junctions by tyrosine phosphorylation, translocates to the nucleus and binds to LEF/TCF family member transcription factors, suggesting a direct link between the adherens junction and subsequent transcriptional regulatory events.

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Dermal Fibroblasts Convert Benzene to Non-Phenolic Compounds that are less Toxic than the Phenolic Metabolites of Benzene

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Benzene is a natural component of petroleum and a major pharmaceutical commodity. Benzene is known to cause systemic disorders via its metabolism in the liver to phenolic compounds. Skin is a significant route of entry of benzene into the body and has remarkable metabolic activity. The metabolism of benzene in the skin has not been reported. Nor are there reports on the effects of benzene metabolites, phenolic or nonphenolic, on skin. We examined (1) the metabolism of benzene in human dermal fibroblasts and (2) the effects of the formed metabolites and the known phenolic metabolites on fibroblast toxicity, apoptosis, lipid peroxidation and interstitial collagenase. The major benzene metabolites, as analyzed by gas chromatography and mass spectrometry, were toluene, its oxidized products (benzaldehyde and benzoic acid), and aniline. With the exception of aniline, these nonphenolic metabolites did not compromise cell viability at concentrations ranging from 1 mM to 160 mM, but induced lipid peroxidation and collagenase. Cell toxicity of the phenolic metabolites, hydroquinone, t-butyl hydroquinone (phenol derivative) and phenol, corresponded to approximate LC50 values, in micro molars, of 30, 50 and 1500, respectively. At concentrations that allowed for greater than 80% cell viability these phenolic compounds induced apoptosis. With the exception of phenol, all the phenolic metabolites induced lipid peroxidation, whereas only phenol induced collagenase. We infer that the skin converts benzene to nonphenolic metabolites that are less toxic and induce oxidative stress response to a lesser degree than the phenolic metabolites of benzene. However, these nonphenolic metabolites can facilitate skin aging by stimulating collagenase expression.

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Overexpression of the 16 kDa N-Terminal Pro-Domain of IL-1α in Basal Layer Keratinocytes Accelerates the Progression Stage of Cutaneous Chemical Carcinogenesis

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Interleukin 1 alpha (IL-1α) is synthesized as an active 31 kDa precursor molecule that accumulates in cells. Keratinocytes have been shown to store significant levels of bioactive pro-IL-1α. Activation of cells by stimulatory molecules, such as inonomycin or LPS, or disruption of the epidermal barrier, results in the extracellular release of the mature 17 kDa C-terminal IL-1α protein. We have previously reported that mice overexpressing this 17 kDa IL-1α protein under the control of the keratin 14 promoter are completely resistant to papilloma and carcinoma induction using the two-stage chemical carcinogenesis model. Cleavage of the 31 kDa precursor also generates a 16-kDa N-terminal pro-piece which remains in the cell. The 16 kDa peptide has a nuclear localization sequence, and transfection of cells with cDNA encoding this molecule induces malignant transformation (Stevenson *et al*, *PNAS* 94:508–513, 1997). To test the transforming potential of the pro-IL-1α protein in cutaneous carcinogenesis, a K14/pro-IL-1α strain of transgenic mice was created and their response to the two-stage chemical carcinogenesis protocol of DMBA and PMA was tested. The kinetics of papilloma formation were enhanced in the pro-IL-1α overexpressing transgenics relative to the wildtype littermate controls, although the maximum number of papillomas per mouse was similar (WT: 13.6 ± 4.2 (n = 11) and Tg: 11.8 ± 7.2 (n = 10). However, the average latency time to carcinoma formation was decreased by 6–8 weeks in the K14/pro-IL-1α Tg mice and the conversion frequency at week 30 postinitiation was 2.5 fold greater than the littermate controls. These results support a role for pro-IL-1α in enhancing tumorigenesis, in particular the well-characterized progression phase of the two-stage model. Further investigation into the biological activity of the 16 kDa pro-IL-1α protein will be required to determine its precise role in cutaneous oncogenesis *in vivo*.

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Macrophage Fusion Up-Regulates N-Acetyl-Glucosaminyltransferase V, β1–6 Branching, and Metastasis in Cloudman S91 Mouse Melanoma Cells

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Macrophage × melanoma fusion hybrids were described with enhanced metastatic potential *in vivo* compared to parental Cloudman S91 cells. These hybrids also showed markedly altered N-glycosylation patterns, and it was proposed that this could be at least one underlying mechanism for enhanced metastasis. N-acetyl-glucosaminyltransferase V (GNT-V) is the enzyme responsible for β1–6 branching of N-glycans with the addition of poly N-acetyllactosamines, and is strongly associated with motility and metastasis in a variety of human cancers, including melanoma. Here we show that GNT-V is up-regulated with regard to both mRNA levels and enzymatic activity specifically in metastatic hybrids as well as parental macrophages, compared to weakly metastatic hybrids and parental melanoma cells. The metastatic hybrids also showed increased binding of the lectin L-PHA which specifically binds the β1,6-branched moiety. In addition, exposure *in vitro* of a cell panel to L-PHA inhibited cAMP-induced motility, specific to metastatic hybrids, suggesting a role for GNT-V in the motility of these cells. Further, there was increased expression of LAMP-1 and β1 integrin, two proteins known to be substrates for GNT-V and also to be associated with metastasis. In summary, macrophage fusion with melanoma cells often causes increased metastatic potential which is associated with enhanced expression of GNT-V and β1,6-branching of N-glycans. These results support the notion that elevated GNT-V in metastases could reflect prior fusion of tumor cells with tumor-infiltrating phagocytes, initiating progression to the metastatic phenotype.

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Control of Survivin Expression in Human Melanomas

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Survivin is an important antiapoptotic protein in cancer research since expression of survivin is correlated with unfavorable prognosis in patients with neuroblastroma, gastric carcinoma, colorectal carcinoma and bladder cancer. Survivin has been found in metastatic and invasive malignant melanomas, however, there are no data on whether survivin expression are associated with melanoma progression, such as the critical switch from radial growth phase (RGP) to vertical growth phase (VGP) or VGP to metastatic stage. In this project we investigated the control of survivin expression during melanoma progression and the mechanism involved. We used Immunoblot to measure survivin expression in melanoma cell lines from RGP melanomas, VGP melanomas and metastatic melanoma. There was higher survivin expression in metastatic melanoma cell lines (average of 8 fold), and in VGP melanoma (average of 3 fold), than in RGP melanoma cell lines. Since deletion of p16 expression has been proposed as a critical event in familial melanomas, and even in early sporadic melanoma, we devised a system for transient transfection of p16−/− melanoma cell lines with a p16 construct to determine whether p16 expression controlled survivin expression. Over-expression of p16 in both RGP and metastatic melanoma lines cells almost completely abolished survivin expression. However, arresting cells at G1/G0 with serum withdrawal and high confluence growth conditions also down-regulated survivin expression, suggesting that control of survivin expression might be more complex and be associated with high proliferation in the cells. We are currently determining whether signaling elements which control other antiapoptotic defenses in melanoma, such as activated Ras or PI3K, affect survivin expression in melanomas. These experiments help understand the expression of survivin in melanomas, and might provide a basis for finding a diagnosis tool for melanoma progression.

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The Chromogen Diaminobenzene Reacts with an Unidentified Substance in *Murine* Melanoma Cells

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The phytoalexin Resveratrol (RES) has been shown to have an inhibitory effect on the growth of *murine* and human melanoma cell lines. Flow cytometric studies using the Annexin-FITC method have shown that RES-treated *murine* melanoma cells undergo characteristic apoptotic membrane alterations. We used the TUNEL method, immunohistochemical (IHC) assays for active caspase 3 and PARP and DNA fragmentation gel electrophoresis to detect additional apoptotic changes in RES-treated B16-F10 *murine* melanoma cells. To detect DNA fragmentation, cells were grown in tissue culture and treated with varying concentrations of RES dissolved in 100% EtOH. DNA was isolated by ethanol precipitation with phenol extraction and examined by agarose gel electrophoresis. The DNA banding pattern of RES-treated melanoma consistently displayed a banding pattern suggestive of apoptosis. For the TUNEL assay and IHC assays cells were grown in tissue culture and implanted by subcutaneous injection into the flank of C57BL mice. The primary tumors that grew in these animals were infiltrated with graded concentrations of RES dissolved in 100% ETOH, grossly dissected and paraffin embedded onto microscope slides. All of the IHC assay techniques utilize a primary antibody, a secondary antibody conjugated to HRP and a developing solution containing the substrate diaminobenzene. Method controls showed that samples treated with only the secondary antibody and DAB developing solution produced a pattern consistent with a positive result. Upon reinvestigation, it was found that the same result could be obtained by treating the samples with the DAB developing solution alone. We feel that these results suggest that *murine* melanoma contains a substance with endogenous peroxidase activity that reacts with DAB. Furthermore, we have shown that this property cannot be quenched by standard H202 pretreatment and therefore chromogenic assays utilizing the diaminobenzene substrate are inadequate for the study of B16-F10 *murine* melanoma. Future investigations will screen developing solutions for a suitable chromogen so that the TUNEL method, antiactive caspase 3 and PARP immunoassays may be used in our model system. Data on chromatin condensation in RES-treated *murine* melanoma using the Hoechst dye technique are currently underway in our laboratory.

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The Novel Tumor Suppressor p33ING1 is Overexpressed but Rarely Mutated in Human Melanoma Cell Lines

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Cutaneous malignant melanoma is a severe life-threatening cancer. Epidemiological evidence indicates that ultraviolet radiation exposure is directly linked to the evident increase of both incidence and mortality rates of melanoma observed in the past years. However, the genetic changes caused by ultraviolet radiation that lead to melanoma formation remain unclear. Although the tumor suppressor gene p53 has been shown to be mutated in over 90% of nonmelanoma skin cancers, its mutation is only observed in 15–25% of melanoma biopsies suggesting that p53 mutation may not be an early step in melanoma development. Recently, a potential tumor suppressor gene p33ING1 was cloned and shown to inhibit cell growth in the presence of p53. ING1 is able to suppress cell proliferation and up-regulate a p53 downstream target gene, p21waf1, leading us to believe that ING1 may be an important tumor suppressor in melanoma formation. To investigate if p33ING1 plays a role in melanoma formation, we examined the p33ING1 protein levels in 14 melanoma cell lines and found that ING1 is overexpressed in 13 of 14 cell lines compared to normal melanocytes. Single-strand conformation polymorphism analysis showed band shifting in two melanoma cell lines. DNA sequencing was performed to verify if p33ING1 was mutated in human malignant melanoma. We detected eight point mutations in exon 2 of the ING1 gene in the SK24 cell line; none were distinctive ultraviolet C to T transitions. Taken together, our data suggests that mutation of p33ING1 is rare in melanoma cell lines.

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Melanocytes do Not Exhibit Directional Migration in a DC Electric Field

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Upon wounding, the endogenous transepithelial battery drives a current out of the wound, generating an electric field of approximately 100 mV per mm at the wound perimeter. Our prior work has demonstrated that when skin-derived keratinocytes are exposed to DC electric fields of this physiologic magnitude, they exhibit galvanotaxis, or directional migration, and we have suggested that this directional migration contributes to wound healing. Since melanocytes must also migrate into the healing wound to repigment it, we examined melanocyte motility during exposure to a DC electric field. Melanocytes were isolated from neonatal foreskins and cultivated in Melanocyte Growth Medium (MGM, Cascade). Cells were plated onto collagen ι-coated coverslips, and placed in an electric field of 100 mV for 2 h in MGM with 2 mM $CaCl_2$. Melanocytes, either exposed to the DC field or nonexposed controls, exhibited similar motilities of 12 μm per h, significantly (3–5 fold) lower than the motility rates of keratinocytes under identical conditions. In addition, melanocytes exhibited no directional migration in the DC field; their migratory paths were random. This is in sharp contrast to contrast to keratinocytes which migrate toward the cathode in an electric field. Dendritic neurons have been reported to lengthen and realign their dendrites in a DC electric field, prompting us to examine the orientation of melanocyte dendrites during electric field exposure. Neither the number of primary dendrites per cell (2–6), nor the orientation of the dendrites with respect to the field vector (random), nor the average length of the dendrites (38 μm) was significantly different in melanocytes exposed to the DC electric field as compared to nonexposed controls. We conclude that in marked contrast to keratinocytes, human skin-derived melanocytes do not respond to DC electric fields of physiologic magnitude with either directional migration or reorientation of dendrites. This may contribute to the delay in repigmentation that often accompanies wound reepithelialization.

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SCF and α-MSH Co-Operatively Drive the Proliferation and Survival of Melanoma 661, a Process Converted to Cell Death by Histamine H1 Receptor Antagonism

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Stem cell factor (SCF) and α-MSH have variable growth effects on metastatic melanoma lines and most often, if an effect is noted, they are growth inhibitory. We have identified a unique melanoma line, 661, that like normal melanocytes, proliferates in response to SCF and α-MSH. The 661 cells are KIT+, HMB-45+ and TRP-1+/− and form tumors in human skin grafts. Melanoma 661 growth *in vitro* is maximized in the presence of serum but the dose–response effect of SCF and a-MSH is most dramatic under serum-free conditions. SCF (50 ?g per ml) induces a 130% increase in cell number after 6 days compared with untreated controls. Similarly α-MSH (10 μM) induces an 80% increase. In cultures with SCF and α-MSH an increase of 200% is noted. Interestingly, the positive growth response to these cytokines can be switched to a negative response if the histamine H1 receptor is blocked with Ketotifen which inhibits 661 growth in a dose responsive manner. In presence of Ketotifen (20 μM) cultures with SCF and α-MSH exhibit a 80% decrease in cell number in 3 days compared with controls (Ketotifen only). Diphenylhydramine (H1 antagonist) can be substituted but not Cimetidine (H2 antagonist). These results suggest that SCF and α-MSH have the capacity to function either as a proliferative/survival factor or a cell death factor dependent on cellular conditions and that this process may in part be modulated through the histamine H1 receptor.

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Atorvastatin Inhibits the Invasive Behavior of Melanoma Cells

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Malignant melanoma is the deadliest skin cancer primarily because of its tendency to metastasize to distant sites. The high metastatic potential of melanoma is thought to result, in part, from enhanced cell motility. Recent evidence suggests that the increased motility of metastatic cells results from activation of proteins belonging to the rho family of small GTPases. HMG CoA Reductase inhibitors prevent the post-translational lipidation of rho proteins, a step essential in their control of cell motility. We tested the hypothesis that disruption of rho activation by the HMG CoA reductase inhibitor, atororvastatin, would block the invasive behavior of melanoma cells. An invasion chamber assay was used to measure the effect of atorvastatin on the motility of A375 melanoma cells. Under the conditions of our assay, A375 cells invaded a model basement membrane while normal melanocytes were unable to do so. Atorvastatin, at a concentration of 4 μM, completely blocked invasion of A375 cells. Moreover, atorvastatin induced a dendritic morphology in A375 cells thereby causing these melanoma cells to resemble non-invasive melanocytes. Atorvastatin, at 40 μM, caused nearly complete killing of the melanoma cells but did not cause death of normal human melanocytes. Tunnel assays showed that the majority of the melanoma cells were dying through apoptosis. Geranylgeranylpyrophosphate blocked both the anti-invasive and apoptotic sequelae of atorvastatin suggesting that these effects resulted from decreased post-translational geranylation. Our results indicate that HMG CoA reductase inhibitors may be effective agents for preventing metastasis and relatively higher doses of these agents might prove to be useful chemotheraputic agents for treatment of melanoma.

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Regulation of Phospholipase D by Protein Kinase C in Human G361 Melanoma Cells

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Regulation of phospholipase D (PLD) by protein kinase C (PKC) isozymes was studied in human G361 melanoma cells. Hydroxyapatite column chromatography and Western blot analysis revealed that G361 cells express PKCβ, δ, ε, ζ but not α. Cells were incubated with [14C]lyso phosphatidylcholine (PC) in the presence of ethanol to label endogenous PC and assayed for release of [14C]phosphatidylethanol after treatment with 100 ng per ml of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for 30 min. In the cells transfected with PKCα, basal level of PLD activity and TPA-induced PLD activation were increased. In the cells transfected with the kinase negative mutant of PKCα, basal level of PLD activity and TPA-induced PLD activation were increased. These results indicate that PLD is regulated by PKCα and kinase activity of PKCα is not necessary for the activation of PLD in G361 melanoma cells. Furthermore, in the cells transfected with PKCε, TPA-induced PLD activation were inhibited, indicating that PLD is differentially regulated by PKCα and PKCε in G361 melanoma cells.

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The Effect of Vascular Endothelial Growth Factor on Primary Human Melanocytes

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Vascular endothelial growth factor (VEGF) regulates endothelial cell proliferation during embryogenesis and tumor formation. It is constitutively produced by keratinocytes but has no known epidermal target cell. We previously reported that normal human melanocytes (Mc) express one VEGF receptor, neuropilin-1. To determine if Mc express other VEGF receptors, Mc were maintained in serum free, hormone and growth factor supplemented medium without phorbol ester or choleragen. By Western blotting, Mc constitutively expressed the tyrosine kinase VEGF receptor 2 (VEGFR-2), in contrast to earlier reports that examined Mc maintained in the presence of phorbol ester. When Mc underwent more than 8 population doublings (~8 weeks) in phorbol ester (50 ng per ml), VEGFR-2 protein expression was lost, emphasizing the importance of studying cultured cells under as physiologic conditions as possible. As this receptor is known to mediate VEGF-induced endothelial cell proliferation and migration, we performed Boyden chamber experiments with Mc and found that VEGF was chemoattractant to Mc and strongly induced their migration. Because UV irradiation is a major modulator of keratinocyte–melanocyte interactions, we examined its effect on Mc VEGFR-2 levels. Within 48 h, UV irradiated Mc had twice the amount of VEGFR-2 compared to sham irradiated cells. Conversely, VEGF (20 ng per ml) decreased the VEGFR-2 level to half of the baseline within 72 h. Tumor necrosis factor-α (TNF-α), which is reported to modulate VEGFR-2 levels in endothelial cells, is also produced by UV irradiated keratinocytes. We observed that TNF-α (40 ng per ml) down-regulated VEGFR-2 in Mc to one-fourth the baseline level within 72 h. The data demonstrate that Mc express VEGFR-2 and migrate in response to VEGF, and that Mc VEGFR-2 expression level is modulated by UV irradiation, VEGF, and TNF-α and thus, Mc may be a target for VEGF of keratinocyte origin in skin.

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CC Chemokine Receptor 7 (CCR7)-Transduced B16 Melanoma Cells Show Enhanced Early Metastases to Regional Lymph Nodes

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CCR7 plays a critical role in the migration of activated dendritic cells from the skin to afferent lymphatics and regional lymph nodes (LN). Both lymphatics and T cell areas of LN constitutively express CCL21, a ligand for CCR7. Since melanoma can metastasize to regional LN via lymphatic channels, we asked whether expression of CCR7 was sufficient to increase the low, spontaneous rate of regional LN metastases by murine B16 melanoma. CCR7 was stably integrated into B16/F1 cells by retroviral gene transduction. By quantitative RT-PCR, CCR7-transduced B16 (CCR7-B16) expressed 1000 fold more mRNA for CCR7 than vector-transduced B16 (Vec-B16). CCR7-B16, but not Vec-B16, showed a Ca^{2+} flux in response to CCL21. After injection of either CCR7- or Vec-B16 cells into the footpads of syngeneic C57/BL6 mice, draining popliteal LN were recovered at 1 and 3 weeks for RT-PCR and gross inspection, respectively. At 1 week when tumors in the footpad were not grossly visible, pooled draining LN from CCR7-B16 injected mice showed at least 200 fold more mRNA for tyrosinase-related protein-1 than did LN from Vec-B16 injected mice in 2 separate experiments. At 3 weeks, 6 of 14 draining LN from CCR7-B16-injected mice (vs. 1/14 LN from Vec-B160-injected mice) showed evidence of metastases. CCR7-B16 LN metastases were larger and sometimes formed multiple colonies within the same LN. Footpad tumor sizes were not different ($n = 14$, $p > 0.5$), and CCL21 did not enhance the growth rate of CCR7-B16 cells in culture. CCR7-B16 LN metastases retained a Ca^{2+} flux response to CCL21 even after 7 passages *in vitro*. Interestingly, 1 of 4 melanoma cell lines initially derived from human LN metastases expressed 10-fold more mRNA for CCR7 than did normal cultured melanocytes. Thus, the expression of a single chemokine receptor gene can increase the early metastases to LN, raising the possibility that cancer cells may co-opt normal mechanisms of LN homing as a means of metastatic dissemination.

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Differential Growth Regulation in Human Melanoma Cell Lines by Tissue Inhibitors of Metalloproteinases-1 and –2

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In addition to inhibiting the proteolytic activity of the matrix metalloproteinases, tissue inhibitors of metalloproteinases (TIMPs) promote the growth of cells in the absence of other exogenous growth factors. In this study, we investigated TIMP-1 and TIMP-2 gene expression and protein production using different malignant melanoma cell lines (MCLs), and examined the role of TIMP-1 and TIMP-2 in the growth regulation of MCLs. Primary MCLs (PM-WK and KHm-4), recurrent primary MCLs (r.p.m.-EP and r.p.m.-MC), lymphnode metastatic MCLs (MM-AN, MM-BP, and MM-RU), and a visceral metastatic MCL (MM-LH) were used. Reverse transcriptase-coupled polymerase chain reaction (RT-PCR) and immunoblotting revealed that all MCLs expressed and produced TIMP-1 and TIMP-2 except for cell line PM-WK, which neither expressed nor produced TIMP-1. TIMP-1 and TIMP-2 levels were measured by enzyme-linked immunosorbent assay (ELISA) in supernatants of cells cultured in the absence or presence of transforming growth factor (TGF)-ƒÀ1, interleukin (IL)-6, and oncostatin M (OSM). In MM-LH and MM-BP, metastatic MCLs which were thought to be cytokine resistant, TGF-ƒÀ1 and IL-6, respectively, up-regulated production of both TIMP-1 and TIMP-2. Moreover, we found that the TIMP-1 production level in MCLs was correlated with the cell migration rate of the MCLs. Cell proliferation was determined by thymidine incorporation after incubation with various concentrations of either TIMP-1 or TIMP-2. The growth of the primary MCLs was stimulated by TIMP-1 and inhibited by TIMP-2. In constast, the growth of the visceral metastatic MCL was stimulated by TIMP-2. We conclude that an altered responsiveness to TIMP-1 and TIMP-2 may be associated with disease progression and acquisition of malignant properties in human melanomas.

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Can Persistent Elevated Wild-Type p53 Expression Explain the Paradox of Low Non-Melanoma Skin Cancer in Vitiligo?

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Previously several case studies reported that patients with vitiligo have a low incidence of actinic damage and nonmelanoma skin cancer (NMSC). This result warranted further systematic investigation in a larger patient population, since these patients lack frequently protective pigment in chronically sun-exposed areas (i.e. neck, head and hands) together with a history of extensive sun sensitivity in association with numerous episodes of sun burns. Considering the onset of the disease in early childhood and adolescence, it would be expected that the cumulative UVR-dose should initiate an increase in sun-related damage (i.e. photodamage, NMSC). Therefore we examined 136 caucasian patients with vitiligo (M/F = 43/93; mean age 42.4 years, skin phototypes II n = 8; III n = 128 Fitzpatrick classification, mean disease duration 17.4 years, positive family history in 66/136). 19 patients received extensive PUVA during their lifetime but none showed lentigines or actinic damage. 16 patients were outdoor workers. Surprisingly, 35% of the entire group (48/136) did not experience sun sensitivity despite a recreational out door life. A thorough examination for photoaging (wrinkles, atrophy, mottled pigmentation, telangectasia, pseudo-scars and laxity) did not show any sign for increased photoaging. Furthermore, there was no case with actinic keratosis (AK) and NMSC. Since p53 is elevated in AK, BCC and SCC as well as in patients treated with PUVA, it was tempting to study p53 expression together with MDM2 and thioredoxin reductase as two representatives of the p53 regulatory system. Surprisingly, we detected a persistent wild-type p53 up-regulation together with down regulation of MDM2 and thioredoxin reductase. There was no increased apoptosis. Our results suggest a direct protective role for wild-type p53 in vitiligo and could explain the low incidence of actinic damage and NMSC in this disease.

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Induction of Invasive Human Melanocytic Neoplasia by Defined Genetic Elements

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Among the three major types of skin cancers, malignant melanoma is the most likely to result in metastasis and death, underscoring the importance of defining factors that induce this cancer. Although forms of melanocytic neoplasia have been developed in transgenic murine tissue models using viral oncogenes, neoplasia is much more easily induced in mouse tissue than human, making generation of a human tissue melanocytic neoplasia from endogenous oncogenes an important goal. To address this, we transduced a mixed population of normal human melanocytes and keratinocytes with a panel of oncogene retrovectors and used them to regenerate human skin on SCID mice. Oncogenes studied alone and in combination via multiplex gene transfer included dominant-negative p53, dominant-negative IkB, CDK4, telomerase (hTERT), cyclin D1 and Ras ($n = 4$ human tissue grafts on SCID mice per vector and vector combination). Of all the combinations of these genes examined, only Ras and hTERT together produced regenerated human skin with clinical and histologic resemblance to malignant melanoma. Unlike the other tested combinations and lacZ controls which entirely lacked any clinical or histologic melanocyte abnormalities, Ras-hTERT skin ($n = 4$) was characterized in all cases clinically by a black plaque occupying the entire human skin graft. Histologically, Ras-hTERT skin tissue displayed massive junctional melanocytic hyperplasia with cellular atypia, abundant mitoses, upward pagetoid spread into suprabasal epidermal layers and dermal invasion of melanocytes through the basement membrane down into the deep dermis to the subcutaneous fat. The keratinocytic component of the epidermis in Ras-hTERT tissue was either entirely normal or only mildly hyperplastic. There was no evidence of metastatic spread in the grafted mice at 8 weeks. These data suggest that interactions between Ras V12 and hTert alone may be sufficient to directly induce features of malignant human melanoma *in vivo* and therefore, may provide both insights into pathogenesis and potential molecular targets for therapy. These findings also establish a general approach to generate human cutaneous neoplasms from defined genetic elements.

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Constitutive Phosphorylation/Inactivation of Retinoblastoma Protein (pRb) by MAPK in Melanoma Cells

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Uncontrolled growth of melanoma cells is mediated in large part by the unrestricted activity of E2F family of transcription factors, responsible for the induction of cell cycle progression genes. Increased E2F transcriptional activity is due to persistent inactivation by phosphorylation of retinoblastoma (pRb) family of proteins (pRb, p107 and p130), eliminating their suppressive association with E2F. Although unregulated cyclin-dependent kinase activity has been implicated in melanomas, the signal transduction pathway and the possible involvement of other kinases have not yet been elucidated. Therefore, we employed AG1024, a specific inhibitor of receptor tyrosine kinase, to investigate the mechanism of pRb inactivation. AG1024 inhibited melanoma cell growth *in vitro* at high efficiency (IC50 less than 50 nM) within 24 h of application. Furthermore, the inhibitor restored tumor suppressive function to pRb within 30 min of exposure, observed by the accumulation of dephosphorylated forms of pRb, p107 and p130, and the formation of growth suppressive DNA binding complexes between E2F and pRb. Surprisingly, the activity of cyclin-dependent kinases (CDKs) was not affected at this early time point. Instead, AG1024 inhibited MAPK activity with similar kinetics as pRb/p107/p130 dephosphorylation. All together, the results suggest that MAPK, a known mediator of cell surface receptor activation, directly neutralizes pRb tumor suppressive function in human melanoma cells probably due to the continuous activity of a receptor tyrosine kinase.

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Role of LEF-1 in Melanoma with Increased Metastatic Potential

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A constitutive complex of β-catenin and LEF-l has been detected in melanoma cell lines expressing either mutant β-catenin or mutant APC (Rubinfeld *et al*, *Science* 275:1790–1792, 1997). However, it has been recently reported that β-catenin mutations are rare in primary malignant melanoma, but its nuclear and/or cytoplasmic localization, a potential indicator of Wnt/β-catenin pathway activation, is frequently observed in melanoma (Rimm *et al*, *Am J Pathol* 154:325–329, 1999). In human malignant melanoma, the appearance of the tumorigenic phase represents a capacity for metastasis and is the significant phenotypic steps in disease progression. Invasive malignant melanoma is proposed to involve at least three steps: (1) capacity to bind the basement membrane; (2) ability to digest the basement membrane; and (3) capacity for cell migration. The current focus of the study is on the latter step, and cell motility is thought to play a crucial role in metastatic behavior. In this work, we sought to determine which LEF/TCF transcription factor of the family was preferentially involved in human melanoma from different stages of tumor progression. We show that LEF-1 mRNA expression is predominant in highly migrating cells from metastatic melanomas. These highly migrating cells showed nuclear accumulation of beta-catenin and active transcription from a reporter plasmid of LEF/TCF binding site. These results may provide a new insight into the role of Wnt/β-catenin signaling pathway in tumor progression of malignant melanoma.

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Flotillin-2 Expresssion Confers Metastatic Behavior to Malignant Melanoma Cells

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Flotillin-2 (Flot-2) is a 42-kDa, highly conserved, caveolae-associated protein we cloned with a monoclonal antibody ECS-1 raised to human keratinocytes. Flotillins have recently been implicated in the pathogenesis of Alzheimer's disease and neuronal regeneration. Over-expression of Flot-2 induced filopodia in cos cells giving them a dendritic, neuron like appearance. Thus, we asked whether expression of Flot-2 differed among cancer cell lines derived from epidermis and neuronal origin. Higher Flot-2 protein was associated with highly metastatic melanoma cell lines compared to nonmetastatic lines. In glioblastoma cell lines higher Flot-2 expression was observed in tumorigenic compared to nontumorigenic lines. To further study Flot-2 in the pathogenesis of metastatic malignant melanoma, we transfected a low tumorigenic, nonmetastatic melanoma cell line (SB2) with green fluorescent protein Flot-2 (pEGFPC2Flot-2) or GFP vector constructs. Stable transfectants were injected into groups of 5 BALB/c nude mice. Two different EGFP Flot-2 transfected SB2 cell lines, but not vector controls, injected subcutaneously, formed tumors as early as 20 days. Flot-2 transfected SB2 cells injected into the lateral vein of nude mice formed lung metastases (range 2–200) in all of the mice in contrast to 3 mice with nontumorigenic control cells (range 0–1) ($F = 109.61$, $p < 0.01$, analysis of variance). Flot-2 has an important role in the tumorigenicity and metastatic behavior of malignant melanoma and may offer new therapeutic avenues for treatment of this deadly malignancy.

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The Alkaloid Noscapine is a Low Toxicity Agent with Significant Antitumor Effects in Melanoma

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Noscapine is an alkaloid derived from opium that has been widely used as a cough suppressant in humans. Our previous studies demonstrated that noscapine binds stoichiometrically to cellular tubulin and thus inhibits normal microtubule assembly. As a result noscapine has a considerable cytotoxic activity against a variety of different types of tumors. We have recently demonstrated that noscapine inhibits melanoma growth *in vivo*. In this study we examined the mechanism by which noscapine is able to inhibit the growth of murine melanoma cells. In addition, we evaluated the toxicity of noscapine *in vitro* and *in vivo* in a murine model to determine its potential use as a chemotherapeutic agent in melanoma therapy. Our results demonstrated that noscapine exhibited potent cytotoxicity effects against murine B16LS9 melanoma cells in a time (0–96 h) and dose-dependent fashion (0–1000 μM) in cell-viability assays. Flow cytometric studies also demonstrated cell cycle perturbations with growth arrest of murine B16LS9 melanoma cells in G2/M phase after noscapine exposure, while normal melanocytes were unaffected. *In vivo*, noscapine treatment of melanoma-bearing C57/BL6 mice did not have any significant hematologic toxicity compared to untreated mice. Additionally other rapidly dividing tissues like gut epithelium, did not exhibit significant toxicity after noscapine treatment. Therefore our results indicate that noscapine has a potent antitumor activity against melanoma cells and demonstrates little toxicity in normal tissue. These studies support the use of noscapine as a potential low toxicity therapy in malignant melanoma.

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Antisense Histone Deacetylase 1 Expression Increases Apoptosis Mediated by Sodium Butyrate in Human Melanoma Cells

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Histone deacetylases are active components of transcriptional corepressor complexes. Inhibition of the histone deacetylase HDAC1 by sodium butyrate (NaBu) or trichostatin A (TSA) results in apoptosis in a variety of normal and tumor cells. Here we demonstrate that inhibition of HDAC1 by millimolar concentrations (1–10 mM) of NaBu results in apoptosis of human metastatic melanoma cells. Apoptosis was preceded by histone H3 and H4 hyperacetylation, p53 acetylation and up-regulation of Bax and down-regulation of Bcl-2 proteins. To determine the specific role of HDAC1 in apoptosis, we generated stable human melanoma cell lines that express sense or antisense HDAC1 vectors. Treatment of HDAC overexpressing cells with NaBu resulted in reduced Bax protein levels and apoptosis compared to cells expressing an empty vector. Conversely, reduced HDAC1 levels (mediated by antisense repression) increased apoptosis mediated by NaBu. Our results are in agreement with recent findings showing that interaction of HDACs with p53 results in its deacetylation, thereby reducing its transcriptional activity. Modulation of HDAC levels could have potential applications for future intervention therapies in the treatment of malignant melanoma.

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Molecular Staging of Melanoma Patients and Analysis of Melanoma Pathogenesis

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Routine histopathological examination of sentinel lymph node (SLN) biopsies provides important staging and prognostic information for patients with clinical stage I-II cutaneous melanoma. Because of its relative insensitivity, however, alternatives to routine histopathology have been sought for evaluation of SLNs. We have adapted a highly sensitive molecular RT-PCR assay to amplify melanoma-related transcripts from fresh-frozen SLNs from 65 patients with clinical stage I-II disease referred to our Multidisciplinary Melanoma Program. Because of variable expression by tumor cells, a panel of 5 transcripts is being studied concurrently: Tyrosinase, GP-100, MAGE-3, MART-1 and MUC18. Positive and negative controls were run in each experiment including B-actin for RNA integrity. All positive RT-PCR results were confirmed by repeat analysis. Seven biopsies were both pathologically and RT-PCR positive. Of the 58 pathologically negative biopsies, 49 were RT-PCR negative whereas 9 were RT-PCR positive. The number of reproducibly positive RT-PCR markers ranged from 2 to 5 for pathologically positive cases and 1–4 for pathologically negative cases. Hence the use of a multimarker RT-PCR is more sensitive than routine pathological examination or single marker RT-PCR. Melanoma pathogenesis is being studied at the transcript level using differential tyrosine kinase display and microarray differential gene expression. Our Northern blot and real-time PCR analyses confirm that DDR2 tyrosine kinase is up-regulated and associated with up-regulation of matrix metalloproteinase-1 expression (an enzyme that specifically cleaves native fibrillar collagen) in HT1080 PMA activated cell line (positive control) and in 2 of 3 melanoma lines relative to normal melanocytes. These findings may have relevance to mechanisms of melanoma invasion and metastasis.

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Multiple Moles: High Frequency of Allelic Deletions at 9p21

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The most important risk factor for the development of malignant melanoma is the total number of benign and atypical melanocytic nevi. Individuals with more than 60 clinically normal nevi harbour a 16 times higher risk for developing melanoma and melanoma risk has been shown to increase steadily with increasing number of benign melanocytic nevi. These findings are based on epidemiologic case control studies. To date however, there are no molecular data supporting these findings. Chromosome 9p21 is a site of frequent chromosomal deletion during melanoma development and allelic deletions within this chromosomal region most often include the tumor suppressor gene p16. We aimed to search for genetic alterations in melanocytic nevi of patients with different numbers of multiple moles and examine possible differences of these genetic alterations in melanoma patients vs. non melanoma patients. According to the nevus number we divided our patients into three groups: patients with less than 20 nevi, 20–60 nevi and more than 60 nevi. Using the microdissection technique, we analysed 7 archived histologically diverse nevi of 5 patients with only a few nevi (< 20), 32 archived nevi of 17 patients with 20–60 nevi and 27 lesions of 14 patients with more than 60 nevi. Using the polymorphic DNA markers D9S171, IFNA and D9S270 we searched for loss of heterozygosity (LOH) at 9p21 within the melanocytic lesions. In patients with < 20 nevi, LOH was detected in 2/7 (29%) of informative cases with at least one marker, in patients with 20–60 nevi in 8/26 (31%) informative cases (6 not informative) and in patients with more than 60 in 12/24 (50%) of cases (three not informative). These data show an increase of LOH with increasing nevus number. Melanoma patients showed LOH in 13/19 (68%) of their lesions (5 not informative) compared to 9/38 (24%) in non melanoma patients (4 not informative) p = 0002. Nevi with melanocytic dysplasia showed LOH in 57% of lesions compared to 35% in benign nevi. Our results show considerable LOH in benign and atypical melanocytic nevi of patients with multiple moles and therefore support the epidemiologic findings of increased melanoma risk by molecular studies. In this subset of patients melanoma patients furthermore show significantly more LOH within their melanocytic lesions than non melanoma patients. Our data

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Loss of Heterozygosity in 6q22–23 in Melanomas and Intradermal Nevi

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Introduction: Chromosome 6 is frequently rearranged in melanomas, with deletions of 6q being observed in 35% to 50% of late-stage melanomas. In particular, rearrangements of the oncogene MYB (located on 6q22) have been observed and postulated to promote late metastasis. Purpose: The objective was to identify new or better genomic markers for predicting risk of nodal metastasis in cutaneous melanoma. Methods: The frequency of 6q22-23 deletion in 19 sporadic melanomas with (11) or without (8) sentinel lymph node involvement and 5 intradermal nevi was examined. Laser capture microdissection (LCM) was used to identify loss of heterozygosity (LOH), using markers D6S1038 and D6S310, in melanocytic cells extracted from the primary lesions. Controls included keratinocytes and lymphocytes from respective patients. Results: In the 24 LCM-enriched tissue samples, 19 out of 24 (79%) were informative for one or more markers. Of these 19, LOH at 6q22-23 was noted in 12 (63%), with no significant difference noted in melanomas with (6/8) or without (3/6) sentinel lymph node involvement or in intradermal nevi (3/5). Conclusions: Using LCM for cell enrichment, our data indicate that 6q22-23 losses may not be related to metastatic spread as previously stated. In contrast, allelic loss at this locus appears to occur very early in the spectrum of melanocytic neoplasia, and is present even in intradermal nevi.

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Distribution of Transforming Growth Factor-1β in Benign and Malignant Melanocytic Neoplasms

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Introduction: Transforming growth factor β (TGF-β) is potent inhibitor of cellular proliferation in a variety of normal cells. Mutations in the TGF-β pathway have been described and correlate with progression to malignant phenotypes in a number of cancers. The purpose of this study is to evaluate the expression of TGF-β1 in malignant melanocytic neoplasms and benign nevi. We hypothesize that malignant melanocytic neoplasms become resistant to growth inhibition by TGF-β1 with a subsequent increase in TGF-β production, which promotes angiogenesis, cell motility and loss of immune surveillance. Materials and Methods: Archival cases were reviewed and representative sections of benign nevi and melanoma were selected. Each specimen was deparaffinized in xylene, followed by serial ethanol dilutions and rehydration. The tissue was incubated with a rabbit ant-TGF-β1 antibody and a secondary anti-rabbit antibody and red chromogen were used for visualization. Positive control was a non-Hodgkins lymphoma and intrinsic smooth muscle. Results: Eleven malignant melanomas, one metastatic lymph node and 14 benign melanocytic neoplasms (compound nevi, intradermal nevi and blue nevi) were studied. Melanomas stained strongly positive for TGF-β1 while benign proliferations were negative or exhibited only weak staining. Conclusion: These results suggest that TGF-β1 may contribute to the mode of progression of malignant melanoma. Production and secretion of TGF-β1 by melanoma cells may have a vital role on suppressing the activation and proliferation of immune modulatory cells.

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Targeted Overexpression of Activated Notch1 Leads to Hair Follicle Abnormalities and "Mohawk Alopecia" in Transgenic Mice

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Notch genes encode a family of highly conserved transmembrane receptor proteins that play a pivotal role in cell fate decisions during vertebrate development. Several Notch genes and their corresponding ligands are expressed in the developing mammalian hair follicle. The aim of our study was to investigate the role of Notch1 in mouse hair follicle development by using a transgenic mouse model. The involucrin promoter was used to drive expression of a gain of function mutation of the Notch1 gene to the inner root sheath (IRS) of the hair follicle. To facilitate analysis of transgene expression, β-galactosidase was expressed simultaneously from the involucrin promoter using an IRES. Transgenic mice displayed both hair and skin abnormalities that can be identified soon after birth. The skin initially appears more wrinkly and scaly and their coats appear more disheveled with wavy hairs. Animals also develop a progressively spreading alopecia that reproducibly spares the midline hair follicles, which we refer to as "mohawk alopecia". Histological examination reveals hyperkeratosis and acanthosis of the epidermis, hair follicles that are abnormally aligned in different orientations, and abnormal differentiation of both the IRS and hair shaft medulla. Analysis of hair follicles from different hair cycle stages reveals defects in hair cycling, with lack of the typical catagen transformations and persistent tricholemmal keratinization.

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Deregulated Activation of the Hedgehog Signaling Pathway Gives Rise to Basaloid Follicular Hamartomas in Adult Mice

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Constitutive activation of Sonic hedgehog (Shh) signaling is a hallmark of basal cell carcinomas (BCCs) and can be caused by loss-of-function *PTCH* mutations or gain-of-function *SMO* mutations, both identified in human BCCs. It is not yet known whether either of these genetic alterations alone, when present in keratinocytes, is sufficient for the formation and maintenance of full-blown BCCs in adults. To begin exploring this issue, we targeted a constitutively active mutant *SMO* allele (*M2SMO*) to mouse skin using a truncated K5 promoter, which is active in a subset of K5-positive cells and can thus bypass lethal phenotypes seen with the full-length K5 promoter. Multiplem δ*K5-M2SMO* transgenic founders exhibited progressive alopecia but, surprisingly, skin tumors were not observed even after one year. Microscopic analysis of skin from adult transgenic mice revealed numerous immature follicle-like structures, anastomosing strands and cords of epithelial cells, and occasional cysts. This histology and additional immunostaining results revealed striking similarities to human basaloid follicular hamartomas, which may be congenital (unlike BCCs) and are frequently associated with alopecia. Northern blot analysis revealed up-regulation of several Shh target genes in transgenic skin, confirming that M2SMO caused constitutive Shh signaling as expected. BCCs were also not seen in an adult transgenic generated using the full-length K5 promoter to drive M2SMO expression, while a *K5-Gli2* transgene produced numerous BCCs in multiple founders and offspring. Proximal activation of Shh signaling in keratinocytes using the human M2SMO mutant, when initiated during mouse embryogenesis, is thus incompatible with BCC development. Moreover, our findings strongly implicate deregulation of the Shh pathway in the genesis of human basaloid follicular hamartomas.

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Anti-Tumor and Tumor-Promoting Capacities of αβ and γδ T Cells in Cutaneous Squamous Cell Carcinoma Development are Dependent on Model of Carcinogenesis

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Squamous cell carcinoma (SCC) of the skin respresents one of the most common malignancies, and chemical carcinogen exposure in the industrual workplace is a major etiologic stimulus. To study the relative roles of αβ and γδ T cells in SCC development, we utilized T cell receptor β and δ knockout (KO) mice on strain backgrounds FVB and C57BL/6, and several modes of SCC induction. Following a single intradermal injection with methylcholanthrene (MCA), FVB βKO and δKO mice were much more susceptible to SCC development than FVB wild-type (w.t.) controls (rel. risk 3.1× for βKO, 3.0× for δKO; $p < 0.02$ for both), indicating that both major subset of T cells exert antitumor responses. Similarly, intradermal injection of PDV, a dimethlybenzathracene (DMBA)-transformed keratinocyte tumor line, induced more carcinomas in both βKO (96.7% sites; 2.3 ± 0.2 week latency) and δKO mice (40.0% sites; 7.0 ± 0.4 week latency) than syngeneic C57BL/6 controls (12.5% sites; 7.3 ± 1.2 week latency). However, strikingly different results were observed utilizing two-stage, DMBA/TPA, chemical carcinogenesis protocols. Under "high-dose" DMBA/TPA exposure, there was a marked *resistance* to tumor (tum) development and carcinoma (ca) progression in FVB βKO (12.7 ± 1.7 tum/mouse and 0.3 ± 0.1 ca/mouse) relative to FVB w.t. controls (20.9 ± 2.0 tum and 2.5 ± 0.4 ca/mouse; $p < 0.0005$ for both tum and ca) – indicating that αβ T cells paradoxically acted in a tumor-promoting capacity. Under "low-dose" DMBA/TPA exposure, more tumors developed in both FVB δKO (13.4 ± 1.3 tum/mouse) and FVB βKOδKO (15.6 ± 1.7 tum/mouse) than FVB w.t. mice (3.1 ± 0.9 tum/mouse; $p < 0.0005$ for both); however, more carcinomas were observed in FVB δKO (3.5 ± 0.5 ca/mouse), but not in FVB βKOδKO (1.3 ± 0.3 ca/mouse), than in FVB w.t. mice (1.3 ± 0.3 ca/mouse; $p < 0.002$ for δKO vs. w.t.). Taken together, these studies demonstrate for the first time: (1) an *in vivo* antitumor function for γδ T cells in three different models of cutaneous carcinogenesis, and (2) the capacity of αβ T cells to promote tumor development and drive progression to carcinoma in two-stage, but not one-stage, carcinogenesis protocols.

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Immunohistochemical Detection for Nuclear β-Catenin in Sporadic Basal Cell Carcimona

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Despite the increasing incidence of basal cell carcinoma (BCC), its pathogenesis has remained largely unknown. Recently, it was reported that genes involved in tissue morphogenesis, like sonic hedgehog or patched, were found to be mutated in BCC, suggesting the involvement of those molecules in the pathogenesis of this tumor. Furthermore, there is evidence that the Wnt-mediated signaling pathway may be one of the downstream targets of sonic hedgehog-mediated signaling, which has led us to focus on molecular events on the Wnt pathway in BCC. Among signal transducers involved in the Wnt pathway, it is clear that β-catenin plays a pivotal role in promotion of morphogenesis and cell growth. In respect to this, it has been reported that, in particular circumstances, as in colorectal cancers, β-catenin migrates to nuclei, where it exerts the ability to activate transcription of various genes. Based on this knowledge, we investigated cellular localization of β-catenin in BCC. Therefore, biopsy specimens derived from BCC (n = 20), from inflammatory skin diseases (n = 10) or from squamous cell carcinoma (n = 5) were immunostained with an antibody directed against β-catenin. Consequently, it was found that 14 of 20 BCC samples tested showed positive nuclear localization of β-catenin, while none of other samples gave rise to positive nuclear staining, indicating nuclear localization of β-catenin as a characteristic feature of BCC, and thereby suggesting its tumorigenic role in this tumor. Hence, this gives us further insight into the molecular pathogenesis of BCC.

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PI3-Kinase/Akt-Mcl1 Siganl Pathway is Involved in IL-6 Regulated Anti-Apoptosis Activity to UVB in BCC Cells

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Interleukin-6 (IL-6) has been shown to be associated with several human tumors. We have established IL-6- over-expressing basal cell carcinoma clone (BCC/IL-6) which is tumorigenic in nude mice. Recent studies demonstrated IL-6 induced Bcl-2 family protein to exhibit antiapoptosis activity to TGF-β through phosphatidyl-inositol 3-kinase (PI3-kinase)/Akt pathway in hepatoma cells. The purpose of the present study therefore was to investigate the PI3-kinase/Akt signaling pathway of IL-6 on the regulation of UVB induced apoptosis in BCC cells. By Western blotting, amount of pAkt and Mcl-1 is found elevated in BCC/IL-6 cells. After treating the BCC cells with IL-6 for 1 h, the amount of pAkt increased 63% comparing with the control BCC cells. After treating the BCC/IL-6 cells with PI 3-kinase inhibitors Wortermannin (100 nM) and LY294002 (25 mM) for 4 h, the amount of pAkt and Mcl-1 decreased to 42.18% and 15.75%, respectively, and, the flow cytometry detected a 40% increment in amount of UVB-induced apoptosis cells. Transient transfection of dominant negative Akt (dnAkt) into BCC/IL-6 cells decrease Mcl-1–17.3% 48 h later. Taking together, these data demonstrated that the PI 3-kinase/Akt-Mcl-1 signaling pathway is involved in IL-6 regulation of UVB induced apoptosis in BCC cells.

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Expression of a Sonic Hedgehog Signal Transducer, HIP (Hedgehog-Interacting Protein), by Human Basal Cell Carcinoma

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Sonic hedgehog (SHH) plays a role in the control of cell differentiation and proliferation in vertebral development and epithelial–mesenchymal interactions. In basal cell nevus syndrome patients, mutations of a gene, patched (PTC), which encodes a receptor for SHH, were found and are thought to be one of the major causes of the disease. Recently, a novel membrane protein, hedgehog-interacting protein (HIP) was found to bind SHH directly and attenuate SHH signaling like PTC, while its expression was induced by SHH signals (*Nature*, 1999). The HIP gene mRNA expression is detected in murine embryonic skin, but it has not been shown in normal human skin or skin tumors. We examined the expression patterns of HIP, SHH, and PTC gene mRNA by human BCCs, in comparison with those by normal human skin and various skin tumors. We found that the mRNA expression of both HIP and PTC genes was enhanced in all samples of BCCs, whereas none of other skin tumors tested exhibited an increased level of such mRNA as compared with that of normal skin. The transcription of SHH gene, however, was in a baseline level in most BCCs. These results indicate that both HIP and PTC gene expressions are specifically involved in the development of BCCs, and that the production of HIP is linked with the expression of PTC but not SHH gene.

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Patched-Smoothened Signaling in Basal Cel Carcinoma

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Basal cell carcinoma induction is tightly linked to Sonic hedgehog pathway dysfunction. Pathway signaling starts at the cell membrane with a complex composed of two proteins: Patched and Smoothened. Patched normally keeps Smoothened inactive. Pathway activation commences when soluble Sonic hedgehog binds to Patched. Sonic hedgehog binding does not result in a physical dissociation of Patched-Smoothened, but it stops Patched from inhibiting Smoothened. Smoothened then up-regulates expression of the transcription factor Gli1. Our preliminary studies using a melanophore based G-protein coupled receptor assay show that Gai is activated by Smoothened: hence Smoothened is a G-protein coupled receptor. Smoothened's Gai activating property and its Gli1 regulating property correlate in three ways: (a) Patched inhibits both activities (b) Sonic hedgehog releases both inhibitions and (c) in the presence of Patched, a mutant Smoothened retains both activities. To provide a more complete understanding of the process by which Smoothened couples to Gai, we are using site directed mutagenesis to investigate specific regions within three of Smoothened's four intracellular domains.

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Gene Expression Portraits of Human Basal Cell Carcinoma using cDNA Microarrays

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Variation in gene expression accounts for much of the biological diversity of human cells and tumors. We propose that the phenotypic diversity of skin tumors results from a corresponding diversity in gene expression patterns, and we have captured these genotypic profiles using cDNA microarray technology. In this study, gene expression portraits of human basal cell carcinoma (BCC) were created using cDNA microarrays spotted with 1700 fully characterized human genes relevant to inflammation, immunity and cancer. BCC tumor specimens from 50 Mohs' Micrographic Surgery patients were compared to their normal skin, and hierarchical gene cluster analysis of the microarray data has revealed novel regulation of numerous families of genes. Certain gene expression patterns are common to all BCCs, while others are specific for BCC histological subtypes (e.g. sclerosing vs. nodular). For example, signaling and angiogenesis activating molecules are up-regulated by greater than two-fold in all BCCs (including ras-related protein RAB-27 A, WEE1-like protein kinase, erbB-3 receptor tyrosine kinase, caspase-8 and endothelin-1). Inflammatory mediators and cell cycle regulatory molecules are down-regulated by greater than two-fold in all BCCs (including interferon regulatory factor 4, prostaglandin-E2 receptor, Lyk tyrosine kinase and G2/mitotic-specific cyclin G1). The following changes appeared specific for sclerosing BCCs: a greater than two-fold up-regulation of proto-oncogene c-crk and melanoma-associated antigen-1 (MAGE-1), and a greater than two-fold down-regulation of integrin α-6 and GM-CSF. In addition, a greater than two-fold down-regulation of IL-6 and interferon-induced proteins appeared specific for nodular BCCs. Collectively, our BCC microarray data significantly contributes to our understanding of UV-induced carcinogenesis and cutaneous tumor biology.

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Ultraviolet Radiation Sensitizes Melanoma Cells to TRAIL-Induced Apoptosis

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Tumor necrosis factor related apoptosis inducing ligand (TRAIL) is a death inducing ligand which was initially described to selectively kill tumor but not normal cells. While the majority of human melanoma cells are highly sensitive to TRAIL, some do not undergo cell death in response to TRAIL. To elucidate the molecular basis of this resistance, we analysed the TRAIL receptor expression of several melanoma lines. All cell lines tested coexpressed the signaling receptors TRAIL-R1 and -R2, whereas one of the resistant lines was deficient in TRAIL-R1. The nonsignalling receptors TRAIL-R3 and -R4 were neither detectable in TRAIL-sensitive nor in resistant melanoma cells. Normal human melanocytes which are resistant to TRAIL also expressed TRAIL-R1 and -R2 but not TRAIL-R3 and -R4. Hence, the differences in TRAIL-sensitivity might not be related to differential receptor expression. Low dose ultraviolet radiation (UV), which by itself did not induce apoptosis, enhanced TRAIL-toxicity significantly. Furthermore, UV rendered resistant melanoma cells highly susceptible to TRAIL. FACS analysis revealed that UV did not influence TRAIL receptor expression. Induction of TRAIL susceptibility by UV was blocked upon inhibition of the death receptor associated protease, caspase-8, while blockade of caspase-9 was less effective. The UV-effect was still observed when UV irradiation was applied up to 6 h before TRAIL exposure. The sensitizing effect of UV was significantly reduced when UV irradiation was performed at 4°C. Since keeping cells at low temperatures during UV exposure is known to inhibit UV-induced death receptor clustering, which is functionally relevant, we hypothesize that UV might render cells susceptible to TRAIL via facilitating death receptor clustering.

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Transgenic Expression of Survivin in Keratinocytes Counteracts UVB-Induced Apoptosis and Cooperates with Loss of p53

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The apoptosis inhibitor survivin has been implicated in both cell cycle control and apoptosis resistance. To investigate apoptotic pathways in keratinocytes in vivo, transgenic mice were generated expressing survivin under the control of a keratin 14 (K14) promoter. K14-survivin mice developed normally, without abnormalities of skin or hair. Basal keratinocyte proliferation in vivo was unaffected. Neither keratinocyte proliferation nor hyperplasia induced by UVB or phorbol ester was enhanced. The generation of sunburn cells in vivo, however, was significantly ($p < 0.01$) reduced in K14-survivin mice compared to non-transgenic littermates. UVB-induced apoptosis of keratinocytes in vitro was blocked by caspase inhibitor ZVAD-fmk, and was reduced in transgenic keratinocytes. By contrast, transgenic keratinocytes were susceptible to apoptosis induced by anti-Fas in vitro. Given the expression of survivin in nonmelanoma skin cancer, we investigated its impact in p53-dependent apoptotic pathways. K14-survivin transgenic mice also deficient in one p53 allele (K14-survivin-p53+/− mice) were significantly ($p < 0.01$) more refractory to sunburn cell formation than either K14-survivin (p53+/+) mice or non-transgenic p53+/− mice, and keratinocytes isolated from K14-survivin-p53+/− mice were as resistant as those from p53−/− mice to UVB-induced apoptosis in vitro. These studies demonstrate that survivin expression in keratinocytes confers resistance to UVB-induced apoptosis without affecting epidermal proliferation or differentiation, and suggest that survivin may counteract the p53-mediated apoptotic pathway in keratinocytes.

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Gadd45 Regulates the G2/M Checkpoint Via cdc2 Kinase, DNA Repair and Cell Death in Mouse Keratinocytes after UV Exposure

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Gadd45 is a p53-dependent, multifunctional protein. We investigated the role of Gadd45 in the UVB response using irradiated normal human and Gadd45 defficient mouse keratinocytes. An RNA protection assay and Western blotting demonstrated that Gadd45 was induced by UVB. Up-regulation of GADD45 mRNA was observed at 4 h after irradiation (100, 300 or 600 J per m^2 UVB) and it remained for 24 h. The induction of Gadd45 protein was observed at 8 h after irradiation and it remained at least for 48 h with the strongest induction at 300 J per m^2. Cell cycle kinetics was assessed using flow cytometry. G2/M and S phase arrest were both observed after exposure to UVB, however, only G2/M arrest was Gadd45 dependent. To establish the mechanism of the G2/M arrest, the activity of cyclin kinase cdc2 was assayed. After immunoprecipitation of cell lysates from irradiated Gadd45+/+ and Gadd45−/− cells with cdc2 antibody, the kinase activity was assessed via histone H1 phosphorylation. While cdc2 kinase activity, thought to be G2 specific, remarkably decreased at 6 h after exposure in Gadd45+/+ cells, the activity was not reduced in Gadd45−/− cells. Post-UV global genomic DNA repair was assessed using the slot-western technique with a thymine dimer specific antibody. DNA from UV-exposed Gadd45+/+ and Gadd45−/− cells was applied on a nitrocellulose membrane, followed by incubation with the thymine dimer antibody. Gadd45−/− cells showed slower nucleotide excision repair than Gadd45+/+ cells. And finally, the cytotoxic effect of UVB was assessed using flow cytometry and crystal violet staining. Gadd45−/− cells showed higher UV sensitivity. Our results suggest that Gadd45 is, at least in part, involved in regulating the G2/M checkpoint after UV exposure, by regulating cdc2 kinase. In addition, Gadd45 enhances global genomic repair and regulates cell death. Gadd45 appears to be an important factor in maintaining genomic integrity in keratinocytes after UV exposure.

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Disruption of Mitochondrial Function During UV-Induced Apoptosis of Human Keratinocytes Requires Activation of Protein Kinase C

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The perturbation of mitochondria structure and function is a critical component of the UV radiation death effector pathway. We investigated the involvement of protein kinase C (PKC) activation in triggering mitochondrial alterations in normal human keratinocytes. In keratinocytes undergoing UV-induced apoptosis, PKCδ is cleaved by caspase-3, generating a constitutively active PKCδ catalytic domain. No other PKC isoforms are cleaved during UV-induced keratinocyte apoptosis. Inhibition of PKC activity protected keratinocytes from UV-induced apoptosis and the loss of cell viability as measured by the MTT assay, which is dependent upon mitochondrial redox reactions. The release of cytochrome c from mitochondria is an early, caspase-independent event in UV apoptosis, and it was not blocked by PKC inhibition. Generation of reactive oxygen species is also an early event in UV signaling, and reactive oxygen species levels were not reduced by PKC inhibition. In contrast, the loss of mitochondrial membrane potential is a late event, and it was blocked by PKC inhibition and the general caspase inhibitor zVAD. The PKCδ catalytic domain generated after UV irradiation was localized to the mitochondria by biochemical fractionation. In addition, ectopically expressed PKCδ catalytic domain induced apoptosis and colocalized with the mitochondrial probe MitoTraker Red by confocal microscopy, strongly suggesting a mitochondrial target for PKCδ. To identify substrates for the mitochondrial PKCδ catalytic domain that may be components of the UV signaling pathway, *in vitro* kinase assays were performed using purified mitochondria and recombinant PKCδ. Several preferred mitochondrial substrates were observed, and the identification of these proteins is under investigation. In summary, these results identify the mitochondria as a functional target for the PKCδ catalytic domain in keratinocytes undergoing UV-induced apoptosis.

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UV-Induced Activation of PI3-Kinase/AKT Pathway is Mediated by both ROS and p38 in Cultured Human Keratinocytes

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Our previous study has shown that as a Ying-Yang balance in response to UV irradiation, PI3-kinase/AKT cell survival pathway was transiently activated, followed by cell death in human skin *in vivo*and in cultured human keratinocytes *in vitro*. The present study focuses on the upstream pathways leading to activation of AKT. Our study using cultured human keratinocytes demonstrates that UV (30 mJ per cm^2) induced phosphorylation of p38 and AKT in a time dependent manner. Phosphorylation of p38 started at 5 min post UV irradiation, peaked at about 30 min, and remained elevated for up to 2 h. Phosphorylation of AKT (Ser473 and 308) occurred at 15 min post UV treatment, peaked at about 1 h, and remained elevated for up to 2 h. We also observed that H_2O_2 induced phosphorylation of both p38 and AKT in a time dependent manner. Pretreatment of human keratinocytes with LY294002 inhibited UV-induced phosphorylation of AKT, suggesting the involvement of PI3-kinase. Pretreatment with NAC, an antioxidant, abrogated UV-induced AKT phosphorylation without apparent effect on p38 phosphorylation, suggesting the involvement of reactive oxygen species in AKT activation in response to UV irradiation. Also,an NADPH oxidase inhibitor, DPI reduced UV-induced AKT activation, suggesting the presence of NADPH oxidase in the activation process. Pretreatment with SB203580, a p38 inhibitor,only partially inhibited UV-induced phosphorylation of AKT, suggesting the role of p38 in AKT activation by UV irradiation. Based on our earlier observations that UV activates Rac1-regualted NADPH oxidase and generates ROS and the data presented here, we conclude that the upstream pathways leading to PI3-kinase/AKT activation preceding the induction of apoptosis due to UV irradiation are mediated by ROS and p38 in cultured human keratinocytes.

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ATR (Ataxia Telangiectasia and Rad3-Related) Mediates a p53-Independent Pathway Required for Survival after UV

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Ultraviolet-induced DNA damage causes cell cycle arrest at G1 and prior to mitosis in order to insure repair in advance of division. ATR has been suggested as essential for damage-induced p53 phosphorylation at serine-15 following UV radiation, and hence implicated as important in p53 activation by UV. Although serine-15 phosphorylation is correlated with activation of p53, study of ATR's true role in the activation of p53 has been hampered by the lack of a means to alter ATR function in cells with an intact p53 pathway (prior studies were carried out in SV40 transformed cells). In order to address this question we generated two stable cell lines with four characteristics: (1) Cells possess an intact p53/G1 response to DNA damage (2) Dominant negative (kinase dead) ATR or wild type ATR can be induced by doxycycline addition (3) No expression of inducible constructs occurs at baseline, and (4) Induced expression of the target allele exceeds endogenous ATR by 3–5 fold. Colony survival assays showed that these cells were 10-fold more sensitive to UV, ionizing radiation or *cis*-platinum after expressing ATR-kDa, while ATR-wt expression had no effect. Although we observed a transient defect in serine-15 phosphorylation of p53 at 3 h after damage, there was no effect of expressing ATR-kDa on damage-induced p53 up-regulation (Western blotting), DNA binding (gel shift), p21 transcription (RNAse protection) or G1 cell cycle arrest (propidium-iodide FACS). These data from cells with wild type p53 lead us to conclude that ATR does not participate in p53 activation as suggested by work done in SV40 transformed cells. Rather, we show that ATR-kDa expression causes cells to improperly proceed into mitosis despite UV damage. This statement is based in part on studies of ATR-kDa expressing synchronized cells which produce mitotic phosphoproteins earlier after UV treatment than control cells. In a separate abstract we report that genetic or pharmacologic interference with ATR function causes massive chromosomal fragmentation and premature chromatin condensation in cells treated with low dose DNA damage suggesting ATR normally blocks mitotic entry in DNA damaged cells. We conclude that ATR plays a role in a p53-independent mitotic entry checkpoint essential for survival after UV and other types of DNA damage.

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Mechanisms of Insulin-Like Growth Factor-1 Receptor-induced Survival of Ultraviolet B Radiation in Keratinocytes

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In the skin, keratinocytes express the insulin-like growth factor-1 receptor (IGF-1R) but they do not synthesize insulin-like growth factor-1 (IGF-1). Dermal fibroblasts support the proliferation of keratinocytes in the epidermis by secreting IGF-1. Similar to what is seen *in vivo*, activation of the IGF-1R in normal keratinocytes grown *in vitro* results in cell growth and replication. However, following the UVB irradiation of cultured normal human keratinocytes, the IGF-1R no longer functions as a mitogenic factor, but actually inhibits further cellular proliferation while promoting cell survival. In order to establish a model system to explore this dichotomy of functions, we sought to determine if the IGF-1R in the immortal human keratinocyte cell line HaCaT functioned similarly to that in normal human keratinocytes. HaCaT keratinocytes were grown in the presence of neutralizing α-IGF-1R antibodies or unrelated α-IgG antibodies before being irradiated with increasing doses of UVB. HaCaT cells with neutralized IGF-1R were found to be 10 times more sensitive to low doses of UVB irradiation than HaCaT cells in which the IGF-1R function was unperturbed. HaCaT keratinocytes were also grown in serum-free M154 keratinocyte medium, containing the full allotment of growth factors, or in M154 medium in which the insulin level was reduced from the normal 5 μg per ml to 50 ng per ml (LoIn medium). HaCaT cells grown in LoIn will only have activated insulin receptors, as 50 ng per ml insulin is insufficient to activate the IGF-1R. HaCaT cells in either M154 or LoIn medium were irradiated with increasing doses of UVB. Once again, HaCaT cells were more sensitive to UVB-induced apoptosis in the absence of IGF-1R (in LoIn medium) at low doses of UVB. These findings indicate that HaCaT cells can be used as a model to study the function of the IGF-1R in response to UVB irradiation. In order to accomplish this goal, we have recently established a genetically modified HaCaT cell line in which the function of the IGF-1R can be selectively inactivated.

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Chemopreventive Effect of the Cyclooxygenase Inhibitor Sulindac Against UVB-Induced Expression of Surrogate Markers of Skin Cancer

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Sulindac is a nonsteroidal anti-inflammatory drug with demonstrated potency as a chemopreventive agent in animal models of carcinogenesis and in patients with familial adenomatous polyposis. In addition, it reduces bradykinin-induced edema in human skin. Since tumor promotion is generally associated with exposure to pro-inflammatory stimuli, it is likely that anti-inflammatory agents may have potent antitumor effects. In this study, we tested the hypothesis that the cylooxygenase inhibitor, sulindac, can protect against UVB-induced injury that is crucial for the induction of cancer. Exposure of SKH-1 hairless mice to a single dose of UVB (240 mJ per cm^2) induces various inflammatory responses including erythema, edema, epidermal hyperplasia, infiltration of polymorphonuclear leukocytes, etc. Topical application of sulindac (1.25–5.0 mg/0.2 ml acetone) to dorsal skin of SKH-1 hairless mice either 2 h prior to or immediately after UVB exposure substantially inhibited these inflammatory responses in a dose-dependent manner. Oral administration of sulindac in drinking water (160ppm) for 15 days prior to and during UVB irradiation similarly reduced these inflammatory responses. These potent anti-inflammatory effects of sulindac suggested the possibility that the drug could inhibit signaling processes that relate to carcinogenic insult by UVB. Accordingly studies were conducted to assess the efficacy of sulindac in attenuating UVB-induced early surrogate molecular markers of photodamage and carcinogenesis. UVB exposure enhanced the expression of p53, c-fos, cyclins D1 and A and PCNA 24 h after irradiation. Treatment of animals with either topical or oral administration of sulindac largely abrogated the expression of these UVB-induced surrogate markers. These results indicate that cyclooxygenase inhibitors such as sulindac are effective in reducing UVB-induced events relevant to carcinogenesis and that this category of drugs may prove to be effective chemopreventive agents for reducing the risk of photocarcinogenesis in human populations.

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Ultraviolet B-Induced Activation of Stat3 is Associated with Epidermal Growth Factor Signaling in HaCaT Cell

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STATs are a class of transcription factors that become activated upon tyrosine phosphorylation. Recent studies have demonstrated that constitutively activated Stat3 signaling directly contributes to oncogenesis by stimulating cell proliferation and preventing apoptosis. Chronic exposure of human skin to ultraviolet (UV) radiation is the major causative factor for skin cancer. We investigated the role of UVB on Stat3 activation and its regulatory mechanisms. Electrophoretic mobility shift assay revealed that constitutive activation of Stat3 was very low in both normal human keratinocyte and an immortalized keratinocyte cell line (HaCaT), whereas three SCC cell lines showed high constitutive activation. A single exposure of HaCaT cells to UVB activated Stat3 in a time- and dose-dependent manner. The activation started at 30 min, peaked at 1 h, and remained activated at least for 6 h following UVB exposure. The elevated DNA-binding returned to the basal level at 12 h. Stat3 activation of HaCaT cell by UVB was reduced dose-dependently by treatment with a tyrosine kinase inhibitor, Genistein. In addition, treatment with an inhibitor of epidermal growth factor (EGF) receptor also down-regulated the UVB-induced Stat3 activation. Finally, pretreatment of HaCaT cells with Genistein increased the UVB-induced apoptosis. These results indicate that Stat3 signaling may have some crucial role for UV-response and it might prevent excessive apoptosis by UV light, and may contribute to skin carcinogenesis.

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Anti-Photocarcinogenic Effects of Baicalein

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Baicalein is an active ingredient from Chinese herb Huang Qin (Scutellaria Baicalensis) and has been shown to have anti-inflammatory and anticancer effects. In this study, the protective effects of baicalein on ultraviolet B radiation (UVB)-induced skin carcinogenesis have been investigated. Baicalein of 1 and 5 μmol in acetone or 0.1% and 0.5% in Lubrajel was applied to SKH-1 female mice prior to escalating doses of UVB radiation ranging from 30 to 90 mJ per cm^2 in two separate experiments. The mice were treated biweekly for a period of 25 weeks. Topical application of baicalein prior to UVB radiation delayed tumor expression as well as overall tumor numbers in a dose-dependent manner in both experiments. Baicalein at 1 and 5 μmol in acetone offere 80% protective effects, and baicalein at 0.1 and 0.5% in Lubrigel showed ~29% and ~44% average inhibition, respectively. Further study revealed that baicalein significantly inhibited UVB-induced oxidative DNA damage as evidenced by substantial reduction of UVB-induced 8-hydroxy deoxyguanosine in an *in vitro* system. In addition, baicalein was shown to down-regulate UVB-mediated phosphorylation of epidermal growth factor receptor and activation of mitogen-activated protein kinases. In conclusion, our studies demonstrate that herbal flavonoid baicalein prevents UVB-induced skin carcinogenesis in hairless mice with inhibition of oxidative DNA damage as anti-initiation, and down-regulation of transduction signaling cascades as antipromotion mechanisms.

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EGF Receptor Transactivation Mediated by the Proteolytic Production of HB-EGF (Heparin-Binding EGF-like Growth Factor) is Essential for TPA-Induced Keratinocyte Migration

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12-O-tetradecanoylphorbol-13-acetate (TPA) regulates various biological functions through PKC activation. In human keratinocytes, TPA induced keratinocyte migration in a dose-dependent manner, with a 2-fold optimum at 10 ng per ml. Recently, it was reported that EGF receptor (EGFR) transactivation was mediated by the proteolytic production of EGFR ligands, such as HB-EGF. Specifically, extracellular stimuli unrelated to EGFR ligands induce shedding of EGFR ligands, by converting the membrane-anchored form of EGFR ligands on the cell surface to the soluble form. Then, released EGFR ligands bind to EGFR, resulting in EGFR activation. This observation led us to hypothesize that TPA-induced keratinocyte migration is mediated by an EGFR transactivation mechanism, since TPA is known to trigger HB-EGF shedding. First, we analyzed the effect of TPA on HB-EGF shedding. Ten ng per ml TPA induced rapid shedding of HB-EGF in a dose-dependent manner within 10–15 min Subsequent maximal activation of EGFR was observed at 15 min OSU8-1, an HB-EGF shedding proteinase inhibitor (*JCB*, 151:209–219, 2000), almost completely inhibited TPA-induced keratinocyte migration as well as HB-EGF shedding at 10 μM. In addition, anti-HB-EGF neutralizing antibody (10 μg per ml) inhibited TPA-induced keratinocyte migration by 75%. To further confirm the role of shed HB-EGF, the effect of CRM197 was examined. CRM197, a nontoxic mutant of diphtheria toxin, specifically binds to the EGF-like domain of HB-EGF and neutralizes the activity of human HB-EGF, but not other EGFR ligands. CRM197 completely inhibited TPA-induced keratinocyte migration at 1 μg per ml. Treatment with anti-EGFR neutralizing antibody (10 μg per ml) and EGFR kinase-specific inhibitor AG1478 (30 nM) inhibited TPA-induced keratinocyte migration by 75 and 100%, respectively. Taken together, these data demonstrate that HB-EGF shedding is essential for TPA-induced keratinocyte migration. This is the first report that EGF receptor transactivation mediated by the proteolytic production of HB-EGF plays a central role in a biologically important phenomenon of keratinocytes.

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Cell Cycle Alterations During Ultraviolet B-Induced Murine Skin Tumorigenesis

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Solar ultraviolet B (UVB) radiation causes extensive cellular DNA damage and the p53 tumor suppressor gene plays a pivotal role in DNA repair, G1 cell cycle arrest, and apoptosis. p53 mutations and dysregulated cell cycle associated proteins have been implicated in the pathogenesis of UVB-induced nonmelanoma skin cancer (NMSC). We have previously shown that in different transformed human epidermoid cell lines lacking p53, UVB induces distinct p53-independent cell cycle alterations. In this study, we evaluated the *in vivo* correlation between cell cycle alteration and skin tumor development in SKH-1 hairless mice exposed to 180 mJ per cm^2 of UVB radiation 5 days a week for 37 weeks in a complete carcinogenesis protocol. Cell cycle analysis of keratinocytes from UVB-irradiated mice showed a gradual increase in S phase cells that correlated with developing epidermal hyperplasia. Mcm5, involved in the initiation of DNA replication, was also increased in the epidermis early in the course of UVB radiation, whereas Cdc6 was induced primarily in papillomas/SCCs. The immunohistochemical distribution of p53 and cyclins A and D1 was confined initially to sporadic single cells and gradually developed into foci of patchy, intense staining in the basal and granular layers of UVB-exposed epidermis. p53 was expressed in all papilloma sections examined, whereas cyclins D1 and A showed 68% and 71% staining in these lesions, respectively. In UVB-induced SCCs p53 was strongly expressed in 90% of the tumors. Patchy staining of cyclin D1 was detected in 55% of lesions, whereas cyclin A staining was limited to 27%. Sequencing analysis of the p53 gene in UVB-induced tumors showed characteristic UVB-induced mutations, comparable to immunohistochemical detection of mutant p53 protein using mutant specific antibodies. Our data indicate that UVB-induced cell cycle dysregulation occurs by multiple mechanisms and that cyclin A and Mcm5, as well as p53 are likely primary targets in NMSC tumorigenesis.

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The B7/CTLA-4 Signaling Pathway Plays a Critical Role in Murine Photocarcinogenesis

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UV radiation (UVR) not only induces skin cancer by its direct mutagenic effects but also by suppressing cellular immunity. Altered B7 costimulation plays a role in UV-induced immunosuppression. Ligation of CD28 by B7 stimulates T cells, whereas B7/CTLA–4 interaction inhibits T cell activation. To investigate B7/CTLA-4 signaling during photocarcinogenesis we irradiated groups of mice with UVB 3x/ week for 6 months. To block B7/CTLA-4 signaling, one group of mice was treated with an antagonistic anti-CTLA-4 Ab i.p. after UVR. Controls were either injected with an irrelevant IgG Ab or left untreated. Chronic UVR induced tumor development in all animals of both control groups. In contrast, in the anti-CTLA-4 Ab treated group only 9 out of 20 mice developed skin tumors. Moreover, anti-CTLA-4 treatment induced strong protective tumor immunity because UV-induced tumors which grew progressively in control mice were rejected upon s.c. transplantation into anti-CTLA-4 treated animals. Since CTLA-4 is able to bind to B7-1 and B7-2 surface molecules, we next used mice deficient in both B7-1 and B7-2 for photocarcinogenesis studies. Accordingly, B7 double-deficient mice showed a significantly reduced skin tumor development. To clarify whether there are differences between the two costimulatory B7 molecules in terms of UV-induced cutaneous carcinogenesis, mice deficient in either B7-1 or B7-2 were chronically UV-exposed. B7-1–/– mice displayed no differences in UV-induced skin tumors compared to the controls. Interestingly, B7-2–/– mice developed UV-induced skin cancer much earlier than the controls. Together, these data indicate that blocking B7/CTLA-4 signaling by anti-CTLA-4 Ab induces immune protection against the development of UV-induced skin tumors. Furthermore, B7-2-mediated costimulation appears to play a more critical role in the protection against photocarcinogenesis than B7-1.

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ATR is Required for a Mitotic Entry Checkpoint that Prevents Premature Chromatin Condensation after UV Exposure

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ATR (Ataxia Telangiectasia and Rad3-related) is homologous to yeast protein kinases required for survival after UV. In a separate abstract we describe the generation of stable human cell lines inducible for wild type or a dominant negative (kinase dead) isoform of ATR. Expression of the kinase dead isoform (ATR-kDa) markedly sensitized cells to multiple types of DNA damage but did not affect p53 function or G1 arrest after damage. Without damage, ATR-kDa had no effect even when expressed for two weeks. In the presence of low dose damage (UV, ionizing radiation or hydroxyurea), ATR-kDa expressing cells underwent profound chromosomal fragmentation as revealed by mitotic spreads. This fragmentation was analogous to that described as ''premature chromatin condensation'' (PCC) induced by caffeine and DNA damage (Schlegel & Pardee, Science, 1986). Caffeine has recently been shown to inhibit ATR protein kinase activity *in vitro*. Indeed, we find *in vivo* that ATR-kDa promoted caffeine's ability to sensitize to DNA damage, while ATR-wt rescued cells from this radiosensitizing effect of caffeine. Cell synchronization experiments showed that ATR is most important in mid-S phase to prevent PCC and apoptosis. Consistent with a presumed role downstream of ATR, adenoviral expression of the kinases Chk-1 or Chk-2 rescued PCC caused by ATR-kDa, while expression of the dominant negative isoforms of these kinases promoted PCC. Because they often lack a DNA damage-responsive G1 checkpoint, cancer cells are often more sensitive to the loss of later cell cycle checkpoints than normal cells. We mimicked the loss of the G1 checkpoint by adenoviral expression of G1/S cyclins (cyclin E or CDK2). Indeed, expression of these cyclins synergized with ATR inhibition to cause increased sensitivity to damage. This result coupled with the sensitivity of S phase cells to ATR inhibition suggests ATR as a possible target for a radiosensitizing agent. We conclude that ATR plays an essential role in a checkpoint which prevents lethal premature mitosis in cells which have received low dose DNA damage.

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The C-Kit Signaling Pathways in Normal and Mastocytosis Mast Cells

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The C-kit receptor is a tyrosine kinase vital in the development and survival of mast cells (MCs). Mutated c-kit autoactivation is associated with the development of adult mastocytosis. Mitogen activated kinases (MEKs) and Janus kinases (JAKs) are pivotal enzymes in c-kit signaling pathways. We examined the roles of JAK-3 and MEKs on the proliferation of mastocytosis cell lines HMC-1 and RMT, which express autoactivated c-kit. When added to these cells for 3 days, the JAK-3 inhibitor WHI-P131 (5 and 20 μM) decreased the number of HMC-1 by 53 $\pm$ 14% and 61 $\pm$ 19% and RMT by 46 $\pm$ 8% and 58% $\pm$ 19%, when compared to controls. The MEKs inhibitor UO126 (5 and 20 μM) also reduced the growth of HMC-1 (47 $\pm$ 7% and 54 $\pm$ 11%) and RMT (23 $\pm$ 9% and 33 $\pm$ 10%). When stem cell factor (SCF, 30 ng per ml) was added to these cultures, it did not significantly reverse the growth inhibiting effects of both WHI-P131 and UO126. Neither inhibitor induced significant cell death in these cultures. We also investigated the role of c-kit on normal skin MCs using *in vitro* MC maturation model. When added to neonatal foreskin cell cultures that containing MC precursors for 7 days, SCF (30 ng per ml) enhanced the number of mature MCs to 34 $\pm$ 15% above controls. The JAK-3 inhibitor did not alter this SCF maturation effect. These results demonstrate that JAK-3 and MEKs are important enzymes in the signaling pathways of auto-activated c-kit. Since their inhibition results in decreased mastocytosis MC proliferation, the primary role of these kinases appears to be in cell division. In contrast, JAK-3 appears play little role in normal skin MC maturation because inhibition of the enzyme did not reduce the number of mature MCs in culture. We conclude that c-kit play an important role in MC proliferation and maturation, but the c-kit signaling pathways for MC proliferation and maturation differ.

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Differences in Proliferation and Maturation Between Normal and Mastocytosis Mast Cells

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We have examined the development of mast cells (MCs) in normal skin and mastocytosis lesions by analyzing the expression of proliferative cell nuclear antigen (PCNA) and the BCL-2 oncoprotein with specific mAbs. MCs were identified with conjugated avidin and mAbs for chymase and tryptase. In normal skin, less than 2% of MCs expressed PCNA and all cells were BCL-2-negative, whereas 30–95% of mastocytosis lesional skin MCs expressed PCNA and 10–45% of these MCs were BCL-2 positive. To confirm these observations *in vitro*, normal skin MCs in culture and the mastocytosis cell lines (HMC-1 and RMT) with activating c-kit mutations were also examined. Normal skin MCs cultured in medium alone had less than 2% PCNA-expression and all of these cells were BCL-2-negative. With the addition of stem cell factor (SCF, 30 ng per ml) to these cells for 7 days, 25% of normal MCs expressed PCNA, but none dividing MCs were observed and the total number of MCs decreased by 8% when compared to the added number of cells at day 0. All these cells remained BCL-2-negative. In contrast, more than 95% of the mastocytosis cell lines expressed both PCNA and BCL-2. The results of this study indicate that mastocytosis MCs with c-kit auto-activation in tumor and culture are highly proliferative and these cells may avoid apoptosis by expressing BCL-2 oncoprotein. In contrast, normal skin MCs in tissue and culture appear to have very low proliferation activity and do not express BCL-2. These latter observations have led us to propose that SCF activation of c-kit in normal skin MCs leads primary to maturation rather than proliferation.

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Folliculotropic Mycosis Fungoides with Central Nervous System Involvement

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Folliculotropic mycosis fungoides is a rare variant of cutaneous T-cell lymphoma, mycosis fungoides (MF) type characterized by atypical lymphocytes preferentially infiltrating the hair follicle epithelium relative to the epidermis. We describe the first case in which laser capture microdissection was used to show that the atypical lymphocytes within the hair follicle epithelium were part of the same tumor clone as those in the epidermis and the patient's blood. This is also the first case of folliculotropic mycosis fungoides with involvement of the central nervous system. The successful procurement and analysis of atypical lymphocytes from hair follicle epithelium by laser capture microscopy ushers in a new era in molecular diagnostics. In reviewing the literature describing atypical lymphocytes infiltrating hair follicle epithelium relative to the epidermis, we encourage the use of the term ''folliculotropic MF.'' Our case also supports previous findings in classic MF patients that CNS involvement can occur in advanced disease.

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Up-Regulated L-Selectin and Diminished Integrin Expression in Sézary Cells: Functional Consequences Under Physiologic Shear Stress Conditions

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Although aberrations in adhesion molecule expression by lymphoma cells have been reported, the functional consequences of these changes are unclear. Herein, we report a patient with Sézary syndrome whose peripheral blood T cells (90%) consisted of a TCRVβ17$^+$ malignant clone as detected by TCRVβ-specific mAb. Thin plaques which showed a dense infiltrate of TCRVβ17$^+$ cells were present on 70% of the patient's body surface. By flow cytometry, peripheral blood malignant T cell adhesion molecule abnormalities included an 80% down-regulation of LFA-1 compared to normal controls and no detectable expression of a4 integrin. Under shear stress conditions (1.5 dynes per cm^2) in a parallel plate flow chamber, malignant T cells failed to arrest on recombinant ICAM-1 in the presence of several chemokines and displayed an 80% decrease in the ability to arrest on TNF-α activated dermal microvascular endothelial cells compared to normal CD4$^+$ memory T cells (mTC). Cutaneous lymphocyte-associated antigen (CLA) expression was detected in ~25% of malignant T cells in the peripheral blood, but was expressed in $<$10% of TCRVβ17$^+$ T cells in the dermis. By contrast, 95% of malignant T cells in peripheral blood expressed L-selectin (CD62L), and (as others have reported with CTCL) L-selectin ligand was detected in dermal blood vessels at affected skin sites by MECA79 mAb. Compared to normal CD4$^+$ mTC, malignant T cells expressed 2-fold more L-selectin and rolled in 6-fold greater numbers/unit area on L-selectin ligand (n = 5, p $<$ 0.0001). Thus, aberrant expression of integrins led to striking defects in Sézary cell arrest on activated endothelial cells *in vitro*. Malignant T cells in this patient, however, entered skin and produced significant clinical disease. We propose that up-regulated expression of L-selectin may be a novel mechanism by which malignant T cells may target skin in greater numbers and compensate for diminished integrin expression.

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Borrelia Burgdorferi is Absent in Cutaneous B-Cell Lymphomas within the United States

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An association between Borrelia burgdorferi and cutaneous B-cell lymphoma (CBCL) has been made in several European countries. The evidence in favor of such an association has recently been based on more definitive tests for the pathogenetic role of Borrelia burgdorferi in CBCL, including positive cultures or PCR amplification of borrelial DNA from lesional skin. However, there has been only one negative report of Borrelia burgdorferi in five North American cases of CBCL. Therefore, we retrieved 38 cases of primary and secondary CBCL from different geographic locations of the United States. Two separate techniques were used to detect borrelial DNA by PCR, a nested PCR method to amplify a Borrelia burgdorferi-specific gene as well as a borrelial chromosomal Ly-1 clone amplification method. Southern blot hybridization was used for confirmation of the PCR results. No Borrelia burgdorferi-specific DNA was detected in any of the 38 CBCL cases, whereas detectable PCR products were obtained with our positive controls. Our findings suggest that Borrelia burgdorferi plays no role in the development or pathogenesis of CBCL in the United States. The findings also suggest that the geographic variation of the clinical manifestations of Borrelia burgdorferi is indeed real and may be secondary to the genetic and phenotypic differences between species present in Europe and North America.