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Comparison of Human Skin or Epidermis Models with Human and Animal Skin in *In Vitro* Percutaneous Absorption

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For the study of *in vitro* skin penetration of candidate drugs, excised animal skin is frequently used as replacement for human skin. Reconstructed human skin or epidermis equivalents have been proposed as alternatives. We compared the penetration properties of human, pig and rat skin with the Graftskin(tm) LSE(tm) (living skin equivalent) and the Skinethic(tm) HRE (human reconstructed epidermis) models using four topical dermatological drugs (salicylic acid, hydrocortisone, clotrimazole and terbinafine) with widely varying polarity. In agreement with published data, pig skin appeared as the most suitable model for human skin: the fluxes through the skin and concentrations in the skin were of the same order of magnitude for both tissues, with differences of at most two- or four-fold, respectively. Graftskin(tm) LSE(tm) provided an adequate barrier to salicylic acid (flux of 45 μg per cm^2 per h as compared to 22 μg per cm^2 per h for human skin), but was very permeable for the more hydrophobic compounds (e.g. about 900-fold higher flux and 50-fold higher skin concentrations of clotrimazole as compared to human skin), even more than rat skin. In the case of the Skinethic(tm) HRE we found similar concentrations of salicylic acid as in human skin (745 μg per g) and an about 7-fold higher flux (153 μg per cm^2 per h). In contrast, the permeation of hydrophobic compounds through the epidermal layer was vastly higher than through split-thickness human skin (up to a factor of about 800). To conclude, currently available reconstituted skin models cannot be regarded as generally useful for *in vitro* penetration studies, while pig skin is a suitable model for human skin for this purpose.

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Altered Sebaceous Gland Morphology and Function in Parathyroid Hormone-Related Protein Transgenic Mice

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Murine transgenic models that either overexpress or lack parathyroid hormone-related protein (PTHrP) in the epidermis suggest that the peptide influences both the development and maintenance of adnexal structures in the adult. In this study we evaluated sebaceous gland morphology and function in both the K14-PTHrP mice and the collagen II-PTHrP (rescued)-PTHrP-knockout mice that overexpress or lack the peptide, respectively. In the adult mouse skin, the most intense PTHrP mRNA and protein labeling was observed in the upper portion of the external root sheath of the hair follicle close to where the sebaceous gland intersects the structure. During the first hair cycle (1–3-weeks), sebaceous gland morphology and function in the two PTHrP transgenic models did not appear to differ greatly from that of wild-type littermates. By 8–12-weeks, differences in coat texture became apparent in both the K14-PTHrP and the rescued PTHrP-knock out mice as compared to sex matched wild-type littermates. The dorsal coat of the 12–16-week-old K14-PTHrP mice appears dry, neutral lipids in the hair were decreased by ~50% and the average sebaceous gland area was increased 27 + 5% as compared to sex matched wild-type littermates. The coat of the 12–16-week-old rescued PTHrP-knockout mice appears to be matted and greasy, neutral lipids in the hair were increased ~2 fold and the average sebaceous gland area was decreased by 53 + 6% as compared to sex matched wild-type littermates. Morphologic analysis of the oldest skin samples available form both of the models (6 months for the rescued PTHrP-knockout and 1.5 years for the K14-PTHrP) suggest that the respective trends in the size of the sebaceous glands appear to become more pronounced with age. Taken together these findings suggest that PTHrP may function to regulate sebocyte precursor proliferation/differentiation in the adult mouse.

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Activators of Peroxisome Proliferator-Activated Receptors (PPARs) Alter the Profile of Lipid Synthesis in Rat Preputial Sebocytes

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Excess of sebum production is one of the major factors contributing to the formation of primary lesions in acne. Androgens play an important role in acne due to their effects on sebaceous gland growth and differentiation. Recently, activators of another subfamily of nuclear receptors, PPARs, have been shown to induce sebocyte differentiation *in vitro* as judged by counting the number of lipid forming colonies stained with Oil Red O. In the present work, we describe the effects of Methyltrienolone (R1881) a specific agonist of the androgen receptor (AR) and selective activators of the three PPAR subtypes (PPARα, PPARδ and PPARγ) on the profile of lipid synthesis in rat preputial sebocyte cultures. Lipid synthesis was analyzed by incorporation of ^{14}C-acetate followed by thin layer chromatography. Polar lipids represented approximately 80%, triglycerides 2–3%, cholesterol 7–8%, fatty acids 2–4%, diglycerides 2%, cholesterol- and wax esters 0–2% of total lipids synthesized by sebocytes. Treatment of sebocytes with the specific AR agonist R1881 at 10^{-8} M did not alter the profile of lipid synthesis. BRL49653, a specific agonist of PPARγ, induced a 2- and 4-fold increase of triglyceride and cholesterol/wax ester synthesis, respectively, while synthesis of diglycerides, cholesterol and fatty acids remained unchanged. A synergistic effect on the triglyceride (5-fold increase) and cholesterol/wax ester synthesis (12-fold increase) was observed when sebocytes were treated with BRL49653 and R1881. The effects of selective agonists of PPARα (Wy14 643) and PPARδ (L-165041) on the lipid synthesis profile of cultured rat preputial sebocytes will also be presented. In conclusion, rat preputial sebocytes respond synergistically to AR and PPAR agonists by an increase in the synthesis of lipids observed in sebaceous glands, namely triglycerides and cholesterol/wax esters.

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The Skin as a Steroidogenic Tissue: Enzymes and Cofactors Involved in the Initial Steps of Steroidogenesis are Expressed in Human Skin and Rat Preputial Sebocytes

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Cytochrome P450 cholesterol side chain cleavage enzyme (P450scc) catalyzes the rate-limiting step in steroid hormone synthesis. In concert with its cofactors, adrenodoxin and adrenodoxin reductase, and transcription factor, steroidogenic factor-1, this enzyme converts cholesterol to pregnenolone. Translocation of cholesterol from the outer to the inner mitochondrial membrane is regulated by the steroidogenic acute regulatory protein. Although the human sebaceous gland can synthesize cholesterol from two carbon fragments and can metabolize steroids such as dehydroepiandrosterone into potent androgens, the de novo production of steroids has not been demonstrated in human skin. The goal of this study is to delineate the steroidogenic pathway upstream from dehydroepiandrosterone by documenting the presence of members of the P450scc system. Immunoreactivity of an antibody to P450scc and cytochrome P450 17-hydroxylase was noted in human facial skin, cultured human sebocytes and keratinocytes, and rat preputial sebocytes. These enzymes were expressed in the epidermis, hair follicles, sebaceous ducts, sebaceous glands, and eccrine glands of human facial skin. Immunoreactivity of antibodies to the steroidogenic acute regulatory protein, adrenodoxin, adrenodoxin reductase and the steroidogenic factor-1 colocalized to areas of cytochrome P450scc and cytochrome P450 17-hydroxylase expression. Results of immunohistochemistry were confirmed with Western blotting using protein extracts of human sebaceous glands. Biochemical activity of cytochrome P450scc and cytochrome P450 17-hydroxylase was documented in rat preputial sebocytes as detected by radioimmunoassay. These data suggest that the skin can synthesize steroid hormones de novo. These steroids in turn may mediate skin diseases such as acne, androgenetic alopecia and hirsutism.

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Human Specific Sebaceous Lipid Synthesis and Sebum Secretion in Human Skin-Scid Mouse Chimera

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Human facial skin from postmenopausal females contains relatively small sebaceous glands mostly associated with sebaceous or vellous hair follicles. Split thickness grafts of such skin, transplanted onto male SCID mice 5–6 weeks of age, revealed presence of small glandular structures within the first two months post transplantation (0.5% of total skin area occupied by glands – TOG). A significant increase in the gland size was noticed between 8 and 12 weeks post transplantation (4.3% TOG). Following this period of time, the glands remained approximately the same size or decreased slightly during the 4–8 weeks. Compared to the skin sample prior to grafting, the glands post transplantation enlarged approximately 6.5 fold. Sebum secretion was first detected by SEBUTAPE four weeks post grafting. There was a gradual increase in sebum secretion that paralleled the observed increase in gland size. Maximal secretion was at 12 weeks. The levels were approximately the same at 20 weeks post grafting. In parallel experiments, when the same skin was transplanted onto female mice, the gland enlargement and sebum production was not observed. However, topical treatment of such grafts with androgens (DHT) resulted in increased gland size and sebum production, similar to that seen in the male mice. Thus, the observed increase in gland size and sebum production was presumably initiated by androgens produced by the male mice. Ex-vivo labeling with 14C acetate, followed by high performance thin layer chromatography (HPTLC), showed increased sebaceous lipid synthesis in 3-month-old transplants when compared to the pretransplantation levels. Interestingly, the sebaceous lipids synthesized in the transplanted skin were human specific, showing the presence of squalene and wax esters and other sebum components, i.e. triglyceride. In addition to human specific lipid production other sebocyte expressed markers, i.e. epithelial membrane antigen, β-endorphin and melanocortin-5 receptor were all detected in the human sebaceous glands in SCID mice. The presented data provide evidence for functional sebaceous glands displaying a human phenotype in the SCID mouse. This

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Metabolic Fate and Selective Utilization of the Major Fatty Acids in Sebaceous Glands

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Wax esters are one of the major products of the lipid secreting sebaceous gland of mammalian skin. The synthetic pathways and enzymes involved have not yet been determined. Towards the understanding of their synthesis, the metabolism of exogenously supplied saturated (16:0, 18:0), monounsaturated, Δ9 (16:1, 18:1) and the linoleic Δ9, 12 (18:2) fatty acids (FAs) was followed in cultured sebaceous glands. Explanted glands from human facial skin and hamster ear were incubated with the above radiolabeled FAs or acetate for 24 h. Acetate and 16:0 were incorporated into all of the cellular and sebaceous specific lipids in both hamster and human systems. The 16:0 was elongated to 18:0 by both species but the hamster glands desaturated them to 16:1 and 18:1, respectively. In both species 16:1 was incorporated more extensively into polar lipids than triglycerides, but not into sebaceous specific lipids and was elongated to 18:1 (Δ11). As proven by HPTLC analysis 18:0 and 18:1 were both incorporated into the cellular lipids but at a lower rate than the 16:0 into wax esters. In the hamster gland 18:0 was desaturated to 18:1 but was not further metabolized to other fatty acids. Furthermore, addition of exogenous 18:1 was not further metabolized. In human explants, linoleic acid was the only fatty acid that appeared to be β-oxidized. That oxidation was specific since it did not induce a general oxidation of other FAs. The ability of the explants to synthesize sebaceous lipids correlated with the putative β-oxidation of linoleic acid. In addition the oxidation of linoleic acid was not detected in hamster explants. Thus the oxidation of linoleic acid is specific for the human sebaceous cells and correlated with their function and differentiation. Our results support the hypothesis that the sebaceous gland selectively utilizes fatty acids.

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Extracellular Protein Binding Diminishes Cellular Uptake, Metabolism and Biological Effect of 13-*cis* Retinoic Acid on Human Sebocytes

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13-*cis* Retinoic acid (13cRA) is rapidly absorbed into human sebocytes, is selectively isomerized to high levels of all-*trans* retinoic acid and exerts an antiproliferative effect by binding the retinoic acid receptors in normal culture conditions *in vitro* (*J Invest Dermatol* 115:321–327, 2000). In this study, we provide evidence that the levels of extracellular binding protein plays an important role for cellular uptake, metabolism and biological effect of 13cRA on human sebocytes. Serum albumin, which selectively binds nonaromatic retinoids, has been used. The addition of highly concentrated bovine serum albumin (20-fold) to the serum-free medium resulted in a decreased and controlled uptake of 13cRA into SZ95 sebocytes, i.e. almost constant levels of 13cRA for 72 h after a single treatment. In addition, delayed isomerization of 13cRA into all-*trans* retinoic acid was observed. The intracellular concentration of all-*trans* retinoic acid after 13cRA treatment was nearly similar to that of 13cRA at 24 h, however, it was 2- to 8-fold higher at 48–120 h after treatment. In a parallel experiment, the antiproliferative effect of 13cRA (10^{-7} M) was diminished by adding high levels of bovine serum albumin in the serum-free medium compared to the effect detected in experiments with normal levels of bovine serum albumin. These results indicate a critical function of serum albumin, as extracellular retinoid-binding protein, in reducing the intracellular concentration of functional retinoids and in diminishing the biological effect of retinoic acids on human sebocytes.

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The Human Sebaceous Gland in Healthy, Non-Lesional and Lesional Acne Skin Expresses Peroxisome Proliferator – Activated Receptor (PPAR) α and β as well as γ

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PPARs are known to be important in fatty acid metabolism and adipocyte differentiation and may play a role in the human sebaceous gland; we have already demonstrated the presence of PPARγ only in the sebaceous and apocrine glands in normal and acne skin by monoclonal antibody. To determine whether there could be a role for other members of the PPAR family, frozen facial skin sections from normal and acne patients and sebocytes cultured from human facial skin were immunostained with antibodies to PPARα and PPARβ. Facial skin cryosections from 5 normal women and 7 acne patients were stained with a goat polyclonal antibody to either PPARα or PPARβ. Positive nuclear staining occurred in basal and early differentiated sebaceous gland cells (but not within fully mature sebocytes) and in the sebokeratinocytes of the pilosebaceous duct. It was also seen in epidermal keratinocytes and the outer root sheath of hair follicles. In acne nonlesional and lesional skin, both sebaceous glands and comedones exhibited positive staining. Primary cell lines of facial sebocytes also exhibited peri-nuclear staining of PPARα and PPARβ. PPARα and PPARβ were more widespread than PPARγ, but were also present in sebaceous glands in normal and acne skin as well as in cultured sebocytes. This suggests that PPARs have a role in sebaceous lipid metabolism in the human sebaceous gland; understanding their role may be important in developing new therapies for the treatment of both seborrhoea and acne vulgaris.

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The Red Deer (Cervus Elaphus) – a New Sebaceous Animal Model which Expresses Both the Androgen Receptor and Peroxisome Proliferator-Activated Receptor γ (PPARγ)

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Red deer skin, like Man, exhibits androgen dependent hair growth, has large sebaceous glands and material is obtainable for research without ethical considerations as animals are routinely harvested for food. Since there are major advantages over laboratory mammals, red deer skin has been investigated to determine its potential as a novel animal model for pilosebaceous research. Winter coat samples of deer neck skin, from 12 to 16 month deer and facial skin samples from women between 20 and 68 years were frozen and sectioned (7 μm). Five sections from each sample were stained with either Sacpic or oil red, to compare and contrast sebaceous gland structure and lipid content or immunostained to identify regions of cell proliferation (Ki67), androgen receptor expression and PPARγ presence. Deer sebaceous glands demonstrated a similar structure and lipid content to human glands, but were smaller in size. The distribution of cell proliferation, androgen receptor expression and PPARγ paralleled that in human glands suggesting that the red deer sebaceous gland could be a useful animal model for sebaceous gland research. Certainly, the presence of androgen receptors and PPARγ suggest that red deer skin is a valuable tissue to study not only the mechanism of androgen action in the sebaceous gland, but also the role of PPARγ in sebaceous gland function.

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Activation of Human Keratinocytes by the LPS-Mimetic, Taxol, is likely Mediated Through Lipopolysaccharide Receptors

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Keratinocytes play a central role in initiating cutaneous inflammation in response to bacterial products, such as lipopolysaccharide (LPS). We have shown previously that the receptors for LPS, such as CD14, and the CD11a,b,c/CD18 family of receptors, are present on human and mouse keratinocytes. Taxol, a potent antitumor agent, is known to stimulate macrophages through the LPS receptors, but which receptors are important for Taxol stimulation are currently debated. We assessed if Taxol similarly could stimulate keratinocytes. Human keratinocyte cell lines, including HaCaT and A431 cells and the telomerase-transduced normal primary keratinocyte line, CL-22, all of which we have previously shown to express LPS receptors, were incubated with LPS or Taxol for 72 h. IL-8 production by the cells in the culture supernatants was determined by ELISA to serve as a measure of cell activation. Taxol stimulated all keratinocyte cell lines, although to a lesser degree than LPS. The different cell types showed similar optimal stimulatory concentrations of Taxol (1–4 ng per ml). However, the ranges of stimulatory Taxol doses varied significantly between the keratinocyte cell lines from 0.1 to 8 ng per ml for HaCaT cells and 0.5–31 ng per ml for A431 cells. Higher concentrations of Taxol did not stimulate these cells to produce cytokines but in fact were toxic, as shown by MTT assays. In contrast, concentrations of Taxol that could stimulate CL-22 cells ranged from 2 to 1000 ng per ml and showed a bimodal distribution of optimal doses of 4 ng per ml and approximately 1000 ng per ml. Curiously, toxic doses of Taxol paralleled the stimulatory concentrations in CL-22 cells leading to cytokine production. This observation may relate to the slower growth kinetics of the CL-22 cells and their relative tolerance to Taxol toxicity compared to the faster growing transformed cell lines. LPS showed no toxicity at any tested concentration as expected. We attempted to see if the keratinocyte responses to Taxol could be blocked by various anti-LPS-receptor antibodies. Although anti-CD14 and anti-CD18 antibodies reduced LPS stimulation in both the HaCaT and CL-22 cells, they only could reduce Taxol stimulation in the HaCaT cell line. However, both the LPS and Taxol responses by the A431 cells appeared to be unaffected by the anti-LPS receptor antibodies. The varied effects of LPS-receptor blockade on these cells may be due to direct signaling through the LPS receptor, TLR-2.

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Expression of CXCR2 and its Major Ligands *In Vitro*: a Functional Model for the Study of Psoriasis

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CXCR2 is an IL-8 receptor on human epidermal keratinocytes. It is not expressed in normal epidermis, but in the differentiated keratinocytes of the psoriatic plaque. Since a role in the disease remains unclear, an *in vitro* model of psoriatic skin expressing CXCR2 was developed, to enable functional studies. Models were constructed by immobilising fibroblasts in a collagen gel, and seeding keratinocytes from the same donor onto the gel surface. Cultures were maintained at the air–liquid interface for 21 days in a fully defined medium. Models derived from normal and psoriatic donors displayed the morphology of *in vivo* epidermis. Immunohistochemistry showed that the distribution of Ki67, basal cytokeratin, cytokeratin 10, involucrin and filaggrin mirrored *in vivo* observations. Importantly, CXCR2 expression was also found to mimic that seen *in vivo*, being absent in normal skin models (n = 3 donors), but strongly expressed in the differentiated keratinocytes of psoriatic models (n = 3 donors). CXCR1, which is not expressed by keratinocytes *in vivo*, was also absent *in vitro*. Distribution of the two major CXCR2 ligands, IL-8 and GRO-α, was identical in psoriatic skin models to *in vivo* psoriasis: both were present throughout the viable keratinocyte layers and absent in the *stratum corneum*. IL-8 levels in conditioned medium from psoriatic models were quantified by ELISA. Peak levels (1.1 ± 0.02 ng per ml) were produced during the highly proliferative phase of model development (day 7), falling to 0.13 ± 0.07 ng per ml by day 21. These studies demonstrated that unique characteristics of psoriatic keratinocytes can be retained *in vitro* in a fully defined, serum-free system. Interactions between CXCR2, IL-8 and GRO-α may play a role in the abnormal differentiation of psoriatic keratinocytes. The model is now being applied to study these interactions, and their implications for psoriasis therapy, using compounds targeted against the receptor.

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Barrier Disruption and UVB Irradiation have an Opposite Effect on Antigen-Presenting Function of Langerhans Cells but a Synergistic Effect on Cytokine Production by Keratinocytes in Mice

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Barrier disruption and UVB irradiation are known to modulate epidermal immunity. It remains unclear whether UVB exposure alleviates or aggravates barrier disrupted inflammatory skin diseases such as atopic dermatitis. To address this issue, we tape-stripped and/or UVB-irradiated earlobes of BALB/c mice, and examined the surface expression of immunocompetent molecules, cytokine expression/production, and antigen-presenting functions of EC from the treated skin. By flow cytometry, tape-stripping and UVB-irradiation augmented and reduced, respectively, the expression of CD54, CD80, CD86 and I-A on Langerhans cells (LC) and the sequential treatments of these two modalities exerted an intermediate effect higher than UVB and lower than tape-stripping. By RT-PCR of EC and ELISA of EC culture supernatants, each of tape-stripping and UVB-irradiation slightly up-regulated or unaffected TNF-α and IL-1α production by EC. However the combination of these treatments markedly increased these cytokine production. Cultivation of EC in the presence of L cells transfected with CD40L augmented the expression of IL-1β, suggesting LC as well as keratinocytes (KC) were stimulated to produce cytokines with tape-stripping plus UVB. Finally, the hapten-presenting ability of LC for trinitrophenyl-immune lymph node T cells was inhibited by UVB and enhanced by tape-stripping, and the combination of these two modalities resulted in intermediate T cell responses, consistent with the data of the surface expression of immunocompetent molecules. These findings suggest that UVB exposure on tape-stripped skin differentially modulates the immune function of LC and KC: UVB oppositely inhibits the upmodulated antigen-presenting ability of LC but further enhances the promoted cytokine production of KC in barrier-disrupted skin.

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α-MSH Peptides Inhibit Cytokine-Stimulated NF-κB/p65 Activation in HaCaT Keratinocytes

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α-melanocyte stimulating hormone (α-MSH), a 13 amino acid peptide derived from the proopiomelanocortin (POMC) precursor, was originally named for an ability to stimulate cutaneous pigmentation. However, studies also document additional roles of anti-inflammation, antipyresis and immunomodulation. In particular, α-MSH is able to inhibit the action of proinflammatory cytokines (e.g. TNF-α) in several tissues including skin. We have previously shown in melanocytes and melanoma cells that α-MSH can inhibit TNF-α stimulated up-regulation of intercellular adhesion molecule-1 and activation of nuclear factor-κB (NF-κB). The aim of this study was to investigate whether the anti-inflammatory ability of α-MSH extends to nonpigmentary skin cells and also to compare the inhibitory potency with other POMC peptides. HaCaT human keratinocytes, which express the melanocortin-1 receptor, were grown *in vitro* and stimulated for 60 min with TNF-α in the presence of α-MSH (10^{-11}–10^{-7} M), MSH 11–13 (L-K-L-P-L-V; 10^{-9}–10^{-5} M), MSH 11–13 (L-K-L-P-D-V; 10^{-13}–10^{-7} M), or adrenocorticotrophic hormone (ACTH; 10^{-13}–10^{-7} M). Peptides were added 15 min prior to TNF-α. NF-κB/p65 activation was assessed by digital image tracking of subunit p65 by immunofluorescence microscopy. α-MSH, MSH L-K-L-P-L-V and MSH L-K-L-P-D-V all significantly inhibited TNF-α stimulated NF-κB activation with maximum inhibition at 37% (10^{-9} M), 44% (10^{-9} M) and 43% (10^{-11} M), respectively (n = 3–5). In contrast, ACTH was found not to be inhibitory. In conclusion, α-MSH and MSH 11–13 tripeptides are effective at inhibiting cytokine activation of NF-κB, supporting previous studies on the anti-inflammatory actions of α-MSH. Present data extends findings to nonpigmentary skin cells.

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The Expression of Epidermal Cytokines by The Change of Epidermal Calcium *In Vivo*

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The skin barrier is composed of corneocytes and intercorneocyte lipid lamellae. Previous research has investigated the direct relation between the epidermal calcium and the proliferation and differentiation of keratinocytes. *In vitro* studies confirmed that calcium could control the proliferation and differentiation of keratinocytes. However, there has been no *in vivo* study due to the absence of an adequate experimental model. We performed this study in order to confirm whether some cytokines are expressed in the epidermis by the change of epidermal calcium without disruption of the skin barrier. Iontophoresis which induces the change of the epidermal calcium gradient without disruption of the skin barrier was applied to the skin of 8–12-week-old hairless mice with a direct current (6 V, 0.8 mA) for 1 h and 2 h. The biopsy specimens were taken at 1, 2 and 3 h after current for RT-PCR, and 1, 3 and 6 h after current for immunohistochemical stain. RT-PCR was performed with the primers of IL-1α, TNF-α and TGF-β using isolated RNA from the separated epidermis. Hairless mice showing acute barrier pertubation with tape stripping were used as a positive control. The density of PCR products was quantitated with an image analyzer. In the immunohistochemical stain, IL-1α, TNF-α and TGF-β were used as primary antibodies. The expression of each cytokines mRNA was increased in the 2 h current condition compared to the 1 h condition. The expression of IL-1α and TNF-α in immunohistochemical stain was increased in iontophoresis skin, but TGF-β was decreased. In conclusion, the change of epidermal calcium may be a direct signal for inducing the expression of epidermal cytokines *in vivo*.

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Activation of Epidermal Growth Factor Receptor Modulates Cytokine and Chemokine Expression in Human Keratinocytes

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Activation of epidermal growth factor receptor (EGFR) provides signals essential to multiple aspects of keratinocyte biology, and a growing number of EGFR ligands have been characterized as autocrine growth factors for keratinocytes. Abnormalities in the expression of EGFR and/or its ligands transforming growth factor (TGF)-α and amphiregulin (AR) have been found in various inflammatory skin disorders, where cytokines such as IL-1 and TNF-α can up-regulate the neosynthesis of EGFR agonists. In this study, we have investigated the capacity of EGFR ligands to modulate the expression of proinflammatory cytokines and chemokines in normal human keratinocytes. Subconfluent keratinocytes were cultured for 24 h in the absence of EGF to allow maximal EGFR expression, prior to stimulation with 1 nM EGF, TGF-α or AR alone, or in association with 100 ng per ml TNF-α or 100 U per ml IFN-γ. A 2–3 fold increase in the amounts of basal IL-1α, IL-1β, IL-1ra, GM-CSF and IL-8 mRNA, and a marked down-regulation of basal RANTES and IP-10 mRNA were evident as early as 2 h following EGFR activation, and were maintained for 24 h. Parallel variations were detected at the protein level by ELISA at 24 h. When used in equimolar concentrations, TGF-α and EGF were equipotent, whereas AR displayed reduced activity. Moreover, EGFR ligands significantly synergized with TNF-α or IFN-γ in the induction of IL-1 members, GM-CSF and IL-8, whereas they counteracted TNF-α or IFN-γ induction of RANTES and IP-10 expression. Preincubation with tyrphostin PD153035, a specific inhibitor of the EGFR tyrosine kinase, markedly attenuated the specific effects of EGFR ligands. Of note, basal levels of RANTES and IP-10 mRNA tended to increase in cultures treated with tyrphostin alone, compared to untreated controls. Our data suggest that EGFR ligands actively participate in the complex networks of keratinocyte activation during inflammatory processes of the skin.

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Physiological Activities of Keratinocyte-Derived Ciliary Neurotrophic Factor in Human Skin and its Pathogenic Involvement in Cutaneous Inflammatory Diseases

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Ciliary neurotrophic factor (CNTF) displayophic activity for parasympathetic, sympathetic, sensory and spinal motoneurons, and for the cell types associated with these neurons at various stages in their development. The purpose of this study was to examine whether human keratinocytes synthesize bioactive CNTF, functional interactions of CNTF with nerve growth factor (NGF), neuropeptides and proinflammatory cytokines, and its pathogenic involvement in cutaneous inflammatory diseases such as atopic dermatitis. Normal human keratinocytes synthesized CNTF as a nonsecreted cytosolic molecule. CNTF stimulated production of NGF from keratinocytes in a dose-dependent manner. While CNTF alone had little effect on the keratinocyte proliferation, CNTF significantly enhanced the trophic activities of NGF to keratinocytes. CNTF induced mast cell degranulation, substance P release from neuronal cells and proliferation of fibroblasts. While stimulatory agents of CNTF synthesis in keratinocytes included substance P, IL-4, IL-6, IL-13, PGE2 and histamine, its suppressive agents were calcitonin gene-related peptide, TNF-α, and eosinophil cationic protein. Lesional skin of atopic dermatitis patients contained a higher amount of CNTF compared with controls. These findings suggest that keratinocyte-derived CNTF interacts with other neurotrophins, cytokines and chemical mediators in human skin, and takes part in the maintenance of skin homeostasis as well as in pathogenesis of some cutaneous disorders such as atopic dermatitis.

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Characterisation of Mouse *Frizzled-3* Expression in Hair Follicle Development and Identification of the Human Homolog in Keratinocytes

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Frizzled genes encode a family of Wnt ligand receptors, which have a conserved cysteine-rich Wnt binding domain and include both transmembrane and secreted forms. Work by others has shown that experimental perturbation of Wnt signaling results in aberrant hair formation, hair growth and hair structure. However, to date there is no information on the contribution of individual Frizzled proteins to hair development. We now report that *Frizzled-3* (*Fzd3*) expression in skin is restricted to the epidermis and to the developing hair follicle. Northern analysis on total mouse skin mRNA revealed a single *Fzd3* transcript of 3.7 kb. RT-PCR and *in situ* hybridization analysis revealed *Fzd3* expression in epidermal and hair follicle keratinocytes. *Fzd3* transcripts are first detected in discrete foci in the developing epidermis of 13 day embryos and later in the hair follicle placodes of 15 day embryos, suggesting a role for this Frizzled isoform in follicle development. Interestingly, in 17 day embryos and 1-day-old newborn mice *Fzd3* expression is limited to suprabasal keratinocytes and is not seen in pelage follicles until 3 days post partum. In 7-day-old neonatal skin, *Fzd3* is expressed throughout the epidermis and in the outer cell layers of hair follicles. These observations suggest a role for Fzd3 signaling in some but not all stages of follicle development. We have also identified the mRNA encoding human *Frizzled-3* (*FZD3*) in epidermal keratinocytes and in the HaCaT keratinocyte cell line. *FZD3* mRNA encodes a 666 amino-acid protein with 97.8% identity to the mouse protein. The *FZD3* gene was mapped using a radiation-hybrid cell line panel to the short arm of chromosome 8 (8p21) between the markers WI-1172 and WI-8496 near the loci for the *Hypotrichosis of Marie Unna* and *Hairless* genes.

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Expression of Frizzled Genes in Developing Hair Follicles

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WNT proteins are secreted signaling molecules that act over short distances to change the fate, shape, movements and proliferation of neighboring cells. Mutations in downstream effector genes of a WNT signaling pathway cause dramatic hair follicle phenotypes, suggesting that interactions of WNT proteins with their receptors, the Frizzled (FZ) proteins, regulate hair follicle formation and hair growth. We have found that Wnts 10b and 10a are expressed in epithelial placode cells at the earliest stage of hair follicle morphogenesis, and that Wnt5a is expressed slightly later in the dermal condensate. To determine whether Fzd genes are also expressed in developing follicles and to identify follicular cells capable of receiving WNT signals, we surveyed for expression of all 10 cloned mouse Fzd genes in embryonic skin. We find that Fzd10 is expressed in the placode and in the dermal condensate as it forms. Fzd1 and Fzd2 are also expressed in the dermal condensate and Fzds 1, 2, 3 and 5 are expressed at low levels in placode cells. As hair follicles develop further, expression of Fzd10, Fzd2 and Fzd1 continues in the epithelial and mesenchymal components of hair follicles, and Fzd5 is expressed in follicular epithelial cells. Expression of Fzd7 begins later in morphogenesis, at the bulbous peg stage, when this gene is expressed in the follicular epithelium. These genes are not expressed in interfollicular epidermis. In contrast, Fzds 3 and 6 are expressed in basal cells of the epidermis as well as in follicular epithelial cells. These results indicate that both epithelial and mesenchymal cells of the developing hair follicle are capable of receiving WNT signals, and that Fzd genes may perform overlapping functions.

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Disruption of WNT–Frizzled Interactions Inhibits Hair Growth

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Communication between different types of hair follicle cells is necessary for the onset of anagen and for normal hair growth, but the signaling molecules operating in hair follicles remain poorly characterized. Several lines of evidence indicate that a paracrine WNT signaling pathway is active in hair follicles at anagen onset and during full anagen. To determine whether genes encoding WNT proteins and their receptors, the Frizzled (FZ) proteins, are expressed in postnatal hair follicles we surveyed for expression of all of the cloned mouse Wnt and Fz genes. We found that Wnts 10a and 10b are specifically expressed in hair follicles at anagen onset. Fz10 is weakly expressed at this stage. Interestingly, Wnts 10a and 10b and Fz10 are also expressed at the earliest (placode) stage of hair follicle morphogenesis, suggesting conservation of the signaling pathways regulating anagen onset and hair follicle morphogenesis. Later in anagen, Wnt3 is expressed in hair shaft precursor cells, adjacent to precortical cells that express Fz7. Wnts 3a, 10a and 10b, and Fz6, are expressed in inner root sheath precursors, and Wnts 11 and 5a, and Fzds 5 and 1, are expressed in subsets of cells in the dermal and outer root sheaths. These results indicate that cells in several different layers of the hair follicle are capable of receiving WNT signals, and suggest that WNTs play multiple roles during anagen. To begin to dissect the functions of different WNT proteins in hair morphogenesis and growth we have made use of endogenous secreted WNT inhibitors that block subsets of WNT–FZ interactions. Ectopic expression of one of these, secreted Frizzled-related protein 2 (sFRP2), in the hair follicle outer root sheaths of transgenic mice causes a phenotype of short hair. We find that this inhibitor does not affect hair follicle morphogenesis or control of the hair growth cycle, but instead alters hair growth during anagen. These results indicate that a subset of WNT–FZ interactions is required for normal hair growth in mature follicles. The functions of other WNT-FZ pairings are likely to be revealed by ectopic expression of other secreted WNT inhibitors.

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Laminin 10 Plays a Critical Role in the Development of Normal Skin and Hair Follicles

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Laminins are important components of all basement membrane and influence a variety of important biological processes including cell attachment and migration as well as tissue development. This study is to evaluate the roles of laminin 10 in normal human skin and hair development. It has been recently found that laminin 10, which contains a newly discovered laminin α5 chain, is produced by human keratinocytes and dermal microvascular endothelial cells. Laminin α5 chain expression is found in normal human keratinocytes, dermal-epidermal junction and hair follicle out root sheet. We used a laminin α5 chain knockout mouse model to study the functions of laminin 10. Due to embryonic mortality of laminin α5 chain gene homozygous knockouts, a full-thickness skin grafting approach, grafting embryonic skins to Nude mice, was used. In this way, grafts can be maintained on the hosts and develop into fully mature adult skins. Interestingly, while grafts of wild-type littermates had normal fur develop, there was no hair formation on the skin grafts of the knockout mice. The skin histological features were examined on the skin grafts before the grafting and 4 weeks after the grafting. The embryo skin of laminin α5 knockouts showed thin but organized stratified epidermis with fewer but normal hair follicles. The skin grafts of laminin α5 deficient revealed hyper- or hypo-keratosis in epidermis and thickened dermis with few immature hair follicles. Electro-microscope analysis identified a discontinuity along basement membrane of dermal-epidermal junction. This data suggests that the α5 chain of laminin 10 may be of importance in skin and later stage hair development. The biological functional studies of laminin 10 α5 chain in skin and hair development are under way both *in vitro* and *in vivo*.

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The Hypothesis of Hair Follicle Predetermination

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In recent years, substantial progress has been achieved in genetic and molecular studies of hair follicle biology. But the molecular data, including expression patterns, cannot be properly appreciated without understanding the basic cellular rearrangements and interactions that maintain hair follicle cyclic transformations. Here, we present a further attempt to understand the interaction between different hair follicle cell populations and outline the major cellular processes that take place during its cyclic progression through periods of growth, regression, and quiescence. The Hypothesis of Hair Follicle Predetermination, as put forth here, represents an extension of the previous models of hair follicle cellular kinetics with one critical modification: the timing of the recruitment of stem cells. The patterns of cell proliferation during anagen-telogen transition, together with morphological evidence, suggest that there are two separate cell populations with proliferation potential in the late telogen hair follicle: the cells of the bulge region (stem cells) and the cells of the hair germ. Further, based on our studies of the hairless phenotype in mice, immunohistochemistry data, and extensive analysis of the recent literature, we provide evidence to suggest that the new HF arises not from the bulge region cells, but instead from germinative cells localized in the hair germ. These germinative cells, in turn, were recruited during the previous hair cycle from the bulge region progeny, which become committed to produce the hair of the next generation. During the subsequent catagen and telogen phases, these cells undergo a process of "maturation", thus acquiring the ability to respond to dermal papilla signaling and produce a new hair. Thus, the activity of hair germ cells is the initiating event of anagen, which precedes the activity of the cells in the bulge region. The hair germ cells give rise only to the ascending part of the growing hair follicle, including the hair shaft and IRS, while the downgrowing ORS is a product of the bulge region progeny. Thus, the mature anagen hair follicle has a dual origin. In conclusion, we suggest that the growth characteristics of the hair follicle and hair shaft are

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Role of TGF-β in the Hair Cycle I: Dissection of Endogenous Cellular Responses

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TGF-β superfamily members direct organogenesis in organisms ranging from humans to flies. The hair follicle provides a tractable system to dissect the function of these potent molecules in mammalian tissue remodeling. We seek to identify endogenous responses to TGF-βs during the hair cycle. The three TGF-βs, TGF-β1, TGF-β2, and TGF-β3 are expressed in or near the hair follicle. Addition of TGF-β2 can increase apoptosis in the outer root sheath of cultured human hair follicles. The best-understood response in other tissues is the phosphorylation of Smads. Anti-phospho-Smad2 antiserum (provided by P. ten Dijke, Netherlands Cancer Institute) detected an antigen that was up-regulated in the outer root sheath of human hair follicles cultured in the presence of TGF-β2. This antiserum showed staining in the outer root sheath during the mouse hair cycle. We also investigated expression of TSC-22 (TGF-β-stimulated clone 22), which can potentiate TGF-β-induced apoptosis in gastric carcinoma cells. TSC-22 antiserum detected an antigen in wild type mouse skin that was absent in TSC-22–/– mice. The dynamic pattern of TSC-22 protein accumulation was distinct from the pattern of antiphospho-Smad2 staining. Thus, it is likely that other signals regulate TSC-22 expression in hair follicles. To test for a role in apoptosis, we compared anti-TSC-22 staining with TUNEL or activated caspase-3 staining (vendor). These data indicated no correlation between the site of TSC-22 staining and the onset of apoptosis during late anagen. Some TSC-22-expressing cells were positive for apoptotic markers. However, strong TSC-22 expression was retained in the dermal papilla and inner root sheath of the catagen hair follicle. We continue to investigate other proposed mediators of TGF-β signaling, to better understand the complex roles of these signals in organogenesis.

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Functional Role of Neutral Endopeptidase (NEP; EC 3.4.24.11) During Rat Hair Cycle

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Various enzyme activities have been reported to change in association with the hair-cycle. We examined the dynamics of neutral endopeptidase (NEP) expression and function during the hair-cycle in rats. NEP activity and its encoding mRNA in the dorsal skins of SD rats were measured throughout the hair-cycle (from 3 to 12 weeks). NEP catalytic activity was elevated at early anagen phase (at weeks 5 and 10) and was decreased during catagen to telogen phases. Simultaneously, changes in NEP mRNA expression were observed in parallel with NEP enzyme activity. Immunostaining of NEP in human scalp skin showed the localization of NEP in the connective tissue sheath in a pattern similar to the distribution of elastic fibers. To determine whether NEP has elastase activity, we measured that catalytic function using STANA or remazol Brilliant Blue labeled insoluble human elastin as synthetic substrates. Intriguingly, we found that elastase activity and NEP activity in human fibroblasts could be completely immunoprecipitated by the NEP antibody. This identity was also corroborated by Western blotting analysis. Further, we cloned NEP cDNA, generated a cDNA expression vector for it and transfected COS cells. The elastase activity of NEP-transfected COS cells was strikingly elevated as was their NEP enzymatic activity. These data suggest that NEP plays an important role in the regulation of the hair–cycle in association with its elastase activity.

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Parathyroid Hormone-Related Protein is Expressed in the Hair Follicle of the Adult Mouse and Appears to Modulate the Hair Cycle

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Murine Transgenic models that overexpressed or lacked epidermal Parathyroid hormone-related protein (PTHrP) manifested complex skin phenotypes with alterations in interfollicular epidermis, dermis and appendages. *In situ* hybridization, RNase protection and immunohistochemistry performed on a series of murine skin samples ranging form E-14 to two years indicate that PTHrP gene expression is largely confined to the hair follicle at all developmental times. In the early samples (from E-14 to E-16), PTHrP transcripts and immunoreactivity were present throughout the hair pegs. At birth and three days PTHrP remained in the outer layers of the upper third of the follicle but was not present in deeper aspects of the structure. At one and two weeks, the peptide was present in the external root sheath both of the upper portion of the follicle as well as the hair bulb. PTHrP gene expression was not observed in a large fraction of resting hair follicles in the adult samples, but could be observed in various stages of anagen hair follicles in a pattern similar to the one and two week-samples. The dynamic expression pattern of PTHrP during the hair cycle and relative lack of expression in the interfollicular epidermis suggest that the hair follicle may be the major functional target for the peptide in postnatal murine skin. Using the K14-PTHrP mouse, we investigated the impact of overexpression of the peptide upon hair growth. The length of all hair types was reduced ~50% and ~25%, respectively, in 3 and 10-week-old K14-PTHrP mice as compared to sex matched wild-type littermates. After morphologically evaluating hair follicles in a two to three-week-old and a wax-stripped adult series of K14-PTHrP mice and littermates, it appeared that reduced hair follicle lengths correlated with a shorter anagen phase in the transgenics. These findings suggest that PTHrP may participate in one of the many signaling cascades that regulate the growth of the murine hair follicle.

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Neurotrophins Differentially Regulate Growth Factor Secretion by Dermal Papilla Cells

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Neurotrophins (NT) accelerate hair follicle (HF) regression (catagen), most likely by stimulating keratinocyte apoptosis via binding to the p75 kDa neurotrophin receptor (*FASEB J* 13:395, 1999; 14:1931, 2000). However, NT may also modulate growth factor secretion by dermal papilla (DP) cells that express high affinity NT receptors TrkB and TrkC during the HF anagen-catagen transition. To test this hypothesis, six matched cultures of human scalp DP cells and dermal fibroblasts were grown for 48 h in the presence of 0.5–50 ng per ml of NGF, BDNF, NT-3 or NT-4. Secretion of different growth factors (SCF, VEGF, TGFβ2, KGF) known to be produced by DP cells during anagen was assessed by ELISA. By immunocytochemistry, both DP cells and dermal fibroblasts express TrkA, TrkC, and p75 kDa neurotrophin receptors. TrkB receptors however, were detected on DP cells but not on dermal fibroblasts. The NT tested had no significant effect on DP cell or dermal fibroblast proliferation. By ELISA, secretion of SCF by DP cells into supernatants was significantly down regulated by BDNF and NT-3, while VEGF secretion was decreased after NT-4 treatment. BDNF also significantly decreased TGFβ2 secretion by DP cells. Secretion of these growth factors by dermal fibroblasts was not affected by NT, suggesting that in addition to TrkB expression on DP, this secretary response to NT may aid the discrimination of interfollicular dermal fibroblasts from DP fibroblasts. These data suggest that NT may modulate HF anagen-catagen transition by altering secretion of DP-derived growth factors that control proliferation, apoptosis and/or differentiation of hair matrix cells.

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***In Vitro* and *In Vivo* Production of Leptin by Human Follicular Papilla Cells and its Regulation by Cytokines and Growth Factors**

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Leptin that is secreted from the adipose tissueÂA plays a crucial role in the regulation of energy balance. Although leptin enhances wound reepithelization and mediates a mitogenic stimulus to human keratinocytes *in vitro,* its biological role in the skin remains unclear. In this study, we examined the production of leptin by hair follicular papilla cells (FP). We isolated 6 FP from human scalp hairs of 2 different donors by the standard microdisection and obtained one clone by limiting dilution. These cell lines and clone displayed an aggregative behavior at a preconfluent density and their expression of α-smooth muscle actin was shown to be stronger than that of an interstitial dermal fibroblast cell (DF) line by Western blotting. In addition, they did not differentiate into adipocytes by the culture medium that could induce preadipocytes into adipocytes. The expression of leptin mRNA by all the FP lines and clone, but not by three different DF lines, was demonstrated with RT-PCR and also with the direct sequencing of their products. Moreover, the culture supernatants of these FP lines and clone, but not those of the DF lines, contained large amounts of leptin protein (300–600 pg per ml), which was revealed by ELISA. The immunoreactivity with polyclonal antileptin antibody was recognized only around the hair bulb area, including the follicular papillae. Finally, when the FP lines were cultured with various cytokines and growth factors, e.g., IL-1β, IL-4, TNF-α, IFN-α, IFN-γ, TGF-β1, bFGF, HGF, KGF, EGF, VEGF, and insulin-like growth factor, the production of leptin was suppressed by IL-4, TNF-α, IFN-α, IFN-γ, TGF-β1, bFGF, or EGF. These data demonstrating the nonadipose tissue production of leptin by FP *in vitro* and *in vivo* and the regulatory roles of inflammatory cytokines and growth factors on its production suggest a crucial role played by leptin in scalp hair growth.

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Role of TGF-β in the Hair Cycle II: Actibation of Caspase-3 and Caspase-9 by TGF-β2

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Hair loss is the result of premature entry into catagen by various causes. In male pattern baldness, we are proposing the involvement of "catagen cascade", in which (1) dihydrotestosterone stimulates TGF-β2 synthesis by dermal papilla cells (DPC), (2) elevated levels of TGF-β2 triggers caspase activation, (3) resulting in the apoptosis of epithelial cells. In the present study, we analyzed the role of TGF-β in the induction of catagen. Human scalp tissues were obtained from plastic surgery. Immunohistochemical study revealed that TGF-β2 but not TGF-β1 or β3 was localized at the boundary area between germinative cells and DPC during the transition phase from late anagen to catagen. In the regressing hair follicle, only TGF-β2 was detected at the epithelial strand. TUNEL-positive apoptotic cells were also observed in the area. To know the role of TGF-β2 in relation to apoptosis, human hair follicles were cultured in the presence of TGF-β2. Using active caspase-9 and −3 specific antibodies, we found that TGF-β2 induced the activation of these caspases in two regions, including the lower part of germinative matrix cells and outer most layer of outer root sheath cells. Dual staining for active caspase-9 and TUNEL demonstrated that active caspase-9 and TUNEL-positive cells were colocalized each other. Active caspase-3 positive cells were detected in the broader area compared to the TUNEL-positive cells. A substance from a plant extract, which was capable to suppress TGF-β action, was effective for hair elongation in the organ culture. Moreover, this substance inhibited caspase activation. Collectively our results suggest that TGF-β2 plays an essential role in the induction of catagen via activation of caspases.

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The RhoA GTPase is Differentially Expressed in the Stem Cell Compartment of the Human Hair Follicle

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We previously showed that human hair follicle stem cells reside in the hair follicle bulge, which is delineated by keratin 15 expression. The purpose of this study was to identify genes which are specifically expressed in the stem cell compartment of the human hair follicle so that we may better understand the molecular requirements for the maintenance of the stem cell phenotype. Hair follicles were plucked from Dispase-treated human scalp skin. Total RNA from telogen hair follicle bulges and from anagen bulbs was labeled and hybridized to cDNA expression arrays (Clontech), which were then developed by autoradiography. RhoA was one of 13 genes present in the stem cell-enriched telogen follicle and not in the anagen bulb. Immunostaining in frozen tissue sections shows that RhoA is coexpressed in the K15 bulge area of both telogen and anagen human hair follicles. In addition, RhoA and K15 expression colocalized in clonogenic keratinocytes, cultured from telogen follicles. Therefore, RhoA appears to be differentially expressed in the keratinocyte stem cells of the human hair follicle. Rho GTPases are small protein switches which mediate a variety of essential cellular responses such as gene transcription, cell-cycle progression, cell adhesion and actin reorganization. Future characterization of RhoA's function within hair follicle stem cells should lead a better understanding of the signaling pathways involved in stem cell maintenance.

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Hair Follicle Involution (Catagen) is Accompanied by Apoptosis-Driven Regression of the Cutaneous Microvasculature

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Recently we have shown that the murine hair cycle offers an excellent model for the study of physiologically occuring angiogenesis during synchronized anagen-development. This physiological angiogenesis contrasts with pathological (e.g. tumor-associated) angiogenesis in that eventually the newly formed blood vessels regress again, typically associated with involution of the perfused tissue. Since we had previously shown that the density of cutaneous microvessels declines during synchronized hair follicle regression (catagen) in mice, we speculated that catagen development is associated with partial regression of the cutaneous microvasculature. Using histomorphometry, double-immunoflourescence staining techniques and transmission electron microscopy, we here demonstrate that apoptosis and degeneration of endothelial cells are indeed detectable during catagen development. This is followed by a decline in the cutaneous microvessel density which is still detectable long after all hair follicles have spontaneously transformed from catagen to the telogen stage of the hair cycle. This sugests that the murine hair cycle not only is idealy suited to study the controls of physiological angiogenesis in mature mammalian skin, but also provides a most instructive and easily accessible model for dissecting the molecular controls that critically regulate blood vessel homeostasis. Murine skin vasculature is clearly altered as a result of primary changes in the hair growth cycle, and appeares to be regulated, at least in part, by factors secreted by the hair follicle itself. Therefore, the apoptosis-driven catagen-associated regression of cutaneous blood vessels in murine skin allows one to dissect the as yet obscure molecular controls that determine normal blood vessel regression. Systematic use of this model may provide novel clues to treatment strategies for, e.g. hemangiomas, vascular malformations as well as for the induction of vascular regression around solid tumors.

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Spatio-Temporal Distribution of Apoptosis in Developing Human Hair Follicles

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Morphogenesis of human hair follicle is a continuous process that has been divided into five morphologic stages: prefollicle, pregerm, hair germ, hair peg, and bulbous peg. In this study, we analyzed the distribution of apoptotic cell death in developing human hair follicle using the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method. In the prefollicle stage, TUNEL-positive cells were identified in the periderm, basal layer keratinocytes and mesenchymal cells. TUNEL-positive cells were seen in the pregerm and hair germ, and condensing mesenchyme around them. In the hair peg stage, TUNEL-positive cells were detected in its outer layer and core cells, presumptive matrix epithelial cells, dermal papilla, and follicle sheath. In bulbous peg stages, TUNEL-positive cells were enriched along hair canals including epidermal, infundibulum, and isthmus portion. In the hair cone area TUNEL-positive cells were identified in the inner root sheath and hair cortex. TUNEL-positive cells were identified in dermal papilla, matrix, and outer root sheath during hair follicle elongation. In sebaceous gland and bulge area, TUNEL-positive cells were also detected. The distribution of apoptotic cells in developing human hair follicles is much more prevalent than in adult. This suggests a generally high cell turnover during embryonic development, in consistence with the highly dynamic tissue remodeling during hair follicle morphogenesis. Apoptosis is associated with specific cell behaviors in at least two aspects. One is association with terminal differentiation of hair keratinocytes. In this case, apoptosis is observed in the keratogenic zone of hair cortex and inner root sheath. This is essential for the formation of hair shaft. The other is association with separation of two tissues. In this case, apoptosis is observed in keratinocytes within the epidermal and subepidermal portion of hair canal. This process is essential to create a space between hair shaft and hair follicle/epidermis so the hair shaft is free to grow out of the skin. In general, the spatio-temporal occurrence of apoptosis is used to sculpt out the morphology of hair follicles.

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p53 Mediates Apoptotic Cell Death During Hair Follicle Regression (Catagen)

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The p53 transcription factor mediates a variety of biological responses including apoptotic cell death. p53 is known to control apoptosis in the hair follicle induced by ionizing radiation and chemotherapy, but its role if any in physiological hair follicle regression (catagen) remains to be elucidated. Here, we show that in C57BL/6 mice p53 protein is strongly expressed and colocalized with TUNEL in the regressing hair follicle compartments during catagen. In contrast to the wild-type mice, p53 knockout mice show significant retardation of catagen ($p < 0.05$) accompanied by the significant decrease in the number of apoptotic cells ($p < 0.01$) in the hair matrix. Furthermore, p53 null hair follicles had altered expression of proteins encoded by p53 target genes that are implicated in the control of catagen (decrease of Bax and insulin-like growth factor binding protein-3, and increase of Bcl-2). These data suggest that p53 is involved in apoptosis control during physiological hair follicle regression and that p53 antagonists may prove useful for the management of hair growth disorders with premature entry into the catagen, such as androgenetic alopecia, alopecia areata, and telogen effluvium.

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p53 and its Target Genes are Essential for Chemotherapy-Induced Hair Loss

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Anticancer drugs induce apoptosis in the hair follicles and hair loss, the most common side-effect of chemotherapy. In a mouse model for chemotherapy-induced hair loss, we demonstrate that p53 is essential for this process: (i) by immunohistology, p53 was up-regulated in the hair follicles after cyclophosphamide treatment, (ii) in contrast to wild type mice, p53-deficient mice show neither hair loss nor apoptosis in the hair follicle keratinocytes that maintained active proliferation after cyclophosphamide treatment, (iii) hair follicles in p53 knockout mice are characterized by down-regulation of Fas and insulin-like growth factor binding protein-3, and increased expression of Bcl-2. To confirm a role for Fas as a p53 target gene in chemotherapy-induced hair loss, we show that Fas knockout mice display significant ($p < 0.05$) retardation of cyclophosphamide-induced hair follicle regression. Furthermore, administration of Fas-ligand neutralizing antibody significantly reduced a number of apoptotic cells in the hair follicle and retarded a rate of hair follicle involution induced by cyclophosphamide. These observations indicate that local pharmacological inhibition of p53 and/or its target genes may be useful to prevent chemotherapy-associated hair loss.

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Fibroblast Growth Factor 5 Inhibits Hair Growth by Blocking Dermal Papilla Cell Activation

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Fibroblast growth factor (FGF)-5 inhibits hair growth and induces catagen in mouse hair follicles *in vivo*. As the receptor for FGF-5 (FGFR1) is expressed in dermal papilla cells (DPCs) that stimulate proliferation of outer root sheath cells (ORSCs), we examined whether FGF-5 attenuated the DPC-stimulated ORSC proliferation using these cells isolated from rat vibrissae. We found, however, that FGF-5 did not directly affect the ability of DPCs to stimulate ORSC proliferation. In contrast, when DPCs prior to their treatment with FGF-5 were activated by FGF-1, a prototype member of FGF family that is expressed in hair follicles during anagen, the ORSC-proliferating activity in the conditioned medium of DPCs was highly enhanced, and FGF-5 clearly attenuated this activity. Thus, the results suggest that FGF-5 inhibits activation of DPCs by FGF-1. We interpret that dermal papillae require activation before they will stimulate hair growth *in vivo*, and that blockage of this activation with FGF-5 results in inhibition of hair growth and induction of catagen.

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Choleratoxin but not Isoproterenol Inhibits Human Hair Follicle Growth *In Vitro*

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Human hair growth requires complex controls that coordinate the proliferation of hair matrix keratinocytes with the differentiation of hair shaft, inner root sheath and outer root sheath keratinocytes. The second messenger cyclic adenosine monophosphate (cAMP) may stimulate keratinocyte growth in low concentrations and inhibit keratinocyte growth while stimulating differentiation in high concentrations. Basal epidermal keratinocytes are known to express β2-adrenoreceptors, and undifferentiated keratinocytes in culture are more susceptible to β2-adrenoreceptor mediated cAMP-induction than differentiated keratinocytes. Here we used immunhistochemistry of human scalp skin samples to investigate β2-adrenoreceptor expression in anagen hair follicles. In addition we tested the impact of the selective β2-adrenoreceptor-agonist Isoproterenol, the selective β2-adrenoreceptor-antagonist propranolol, and nonselective cAMP induction by choleratoxin on human hair growth *in vitro*, utilizing cultured androgen-insensitive scalp hair follicles. We found strong and selective β2-adrenoreceptor expression in outer root sheath keratinocytes of the dermal compartment in the isthmus and bulge region and weaker expression in selected layers of the proximal inner and outer root sheath, but not in matrix keratinocytes. However, 10^{-6}, 10^{-9}, or 10^{-12} M isoproterenol or propranolol showed no significant modulatory effect on human hair follicle growth when compared to controls. In contrast, choleratoxin significantly inhibited hair growth at 10^{-8} and 10^{-9} M. Thus, while β2-adrenoreceptor stimulation in the inner and outer root sheath does not appear to alter hair growth, strong nonselective cAMP-up-regulation by choleratoxin, e.g. in the hair matrix, may stimulate premature keratinocyte differentiation in the hair follicle, thus inhibiting hair growth. This suggests that targeting second messenger systems like cAMP is a promising strategy for the manipulation of human hair growth.

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Hair Growth Inhibition by Prolactin and Expression Pattern of Prolactin and its Receptor in Isolated Human Hair Follicles

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The maternity hormone prolactin (PRL) has recently been implicated in hair growth regulation, is expressed also in extrapituitary tissues, and exerts a wide variety of growth-regulatory effects. Recently, we have shown that prolactin is expressed in a hair cycle-dependent manner in mice and that treatment of anagen hair follicles with prolactin resulted in catagen-induction in murine skin organ culture. Here, we provide the first study of prolactin and prolactin receptor expression in human hair follicles (HF), and study the influence of prolactin on hair growth in human follicles. Human HF from scalp hair transplants were isolated and cultured for up to eight days in Williams E medium and prolactin (20 ng, 200 ng, 400 ng per ml) was added every other day. Hair shaft elongation was measured and photodocumented at regular intervals. By immunohistochemistry, prolactin immunoreactivity (IR) was detected in human anagen VI HF in the inner root sheath (IRS), matrix cells of the hair bulb, and outer root sheath (ORS). The dermal papilla fibroblasts were negative. Prolactin receptor IR showed a very similar pattern, as it was detected in the ORS, proximal IRS, and in most of the matrix cells. The dermal papilla and distal IRS of anagen VI HF were negative for prolactin receptor IR. In catagen HF, prolactin and PRL receptor expression appeared to be up-regulated. ORS and matrix cells showed IR, while in the dermal papilla and IRS no prolactin expression could be detected. Treatment of human HF with high dose of prolactin (400 ng per ml) resulted in significant inhibition of hair growth and induction of a catagen-like HF transformation. Ki-67/TUNEL double-staining showed a significantly reduced number of proliferating and increased number of apoptotic cells in the hair bulb of PRL-treated HF, compared to vehicle controls. These data support the hypothesis that the human HF is a source and target of PRL, that PRL is an autocrine hair growth modulator which inhibits proliferation and induces apoptosis in HF keratinocytes, and raises the question whether PRL is involved in the pathogenesis of androgenetic alopecia, at least in females.

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The Potassium Channel Openers Minoxidil and Diazoxide Stimulate Hair Growth in Whole Organ Culture

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Although potassium channel openers such as minoxidil and diazoxide promote hair growth *in vivo*, the mechanisms by which they do so are unclear. Currently, there is no readily available model system since human skin is limited. Therefore, we have investigated the effects of varying concentrations of minoxidil and diazoxide in a red deer whole hair follicle culture system. Anagen follicles were microdissected from the mane region of 10 adult stags at the same time of year, washed with sterile phosphate buffered saline and incubated in Williams medium E with 5 mM glucose, 100 U per ml penicillin and 2.5 μg per ml amphotericin B. A minimum of six follicles were incubated in each experimental group; control vehicle (0.01% dimethylsulphoxide), 0.1, 1, 10, 100 μM minoxidil or diazoxide. Growth was measured by micrometry every 24 h. Red deer follicles grew under all conditions for up to 8 days. All concentrations of minoxidil (5 animals) significantly ($p < 0.01$) increased follicle length compared to the control. Diazoxide (5 animals) had no significant effect, although 0.1 and 10 μM tended to increase growth. The *in vitro* response of the potassium channel openers in this study reflect the clinical response to these drugs. These results confirm deer follicles as a system to examine the mechanism of action of potassium channel openers and assess the effects of other stimulators or inhibitors of hair growth *in vitro*.

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Remodeling of Desmosome (DS) and Hemidesmosome (HD) During Human Hair Follicle Development

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Major cell attachment structures of epidermal keratinocytes, DS and HD, are thought to play important roles in regulation of cell growth and differentiation during hair follicle morphogenesis. We investigated the expression of major components of DS and HD including desmogleins, desmoplakins, plakoglobin, laminin 5, β4 integrin, BPAG1 & 2, plectin, in the developing human hair follicles of skin samples from human fetuses of a series of estimated gestational ages (EGAs) (65–163 days EGA). In the hair germ (65–84 days EGA) and the hair peg (85–104 days EGA), the inner cells being not in contact with the basement membrane zone (BMZ) showed strong membranous immunoreactivities for DS components. Positive stainings for HD components were continuously observed in BMZ of the hair germ and the hair peg, although, in lower portion of the hair peg, the stainings were weak or negative. In the bulbous hair peg (105–135 days EGA) and the differentiated lanugo hair follicle (135 days EGA), the DS components were expressed in the entire hair follicle, although the expression was weak in the outermost layer of the outer root sheath, the bulge cells and the matrix cells. The HD components were expressed in BMZ continuously from the interfollicular epidermis to the mid portion between the bulge and the bulb, and also in the contact area between the matrix cells and the dermal papilla cells. The HD components were absent in the bulbar region under the mid point between the bulge and the bulb. Ultrastructurally, formation of DS and HD was confirmed at the expression sites of their components. These findings indicated that expression of DS components was weak in the cells in contact with BMZ and that expression of HD components was weak or absent in the elongation site of developing hair follicles, all the time during human hair follicle morphogenesis.

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The Type I Keratin 17 is Crucial to the Formation and Structural Rigidity of the Hair

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During development onset of keratin 17 (K17) synthesis marks the adoption of an appendageal fate within the embryonic ectoderm. In adult skin, K17 persists in specific subset of epithelial cells within hair, glands, and nail. The K17 gene is induced following epidermal injury, in inflammatory lesions and basal cell carcinoma. Inherited mutations in K17 can cause either type 2 pachyonychia congenita or steatocystoma multiplex, two distinct ectodermal dysplasias. We inactivated the mouse keratin 17 gene through gene targeting and homologous recombination to define its function(s) in skin epithelia. A subset of K17 null mice show a striking delay in the postnatal emergence of the fur coat. This phenotype appears during the first week postbirth and correlates with the appearance, as seen through histology, of melanin pigment aggregates, destruction of the inner and outer root sheaths, and of twists and breaks in hair shafts. These structural aberrations in the hair were confirmed by electron microscopy. Hair breaks occurred spontaneously during the *ex vivo* manipulation of hairs from K17 null but not wildtype mice. The hair phenotype normalizes to a significant extent in many of these mice with the onset of the first postnatal hair cycle. Nail structure and histology is comparatively normal. While such hair alterations are seen in two independent transgenic lines, the phenotype is not fully penetrant in the original mixed genetic background. Normal-looking K17 null mice exhibit an increased amount of K16 protein in hair extracts when compared to either phenotypic K17 null mice or wildtype mice, raising the possibility that this related keratin gene, which is also wound-inducible, could compensate for the absence of K17. Preliminary studies confirm that the impact of the K17 null mutations depends upon the genetic background, a finding that could have important implications for our understanding of the variable phenotype of patients whose genome harbor similar K17 mutations. These findings point to a very critical role for the K17 protein in the formation and structural rigidity of the hair.

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Overexpression of Nitric Oxide Synthase in the Skin of Transgenic Mice

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Nitric oxide (NO) is produced in cells by nitric oxide synthase. Low concentrations of NO serve as a signaling molecule while high concentrations lead to apoptosis and cytotoxicity. We have hypothesized that high concentrations in the skin may be manifest as GVH, toxic epidermal necrolysis, scleroderma, alopecia areata, graying of hair and vitiligo. Transgenic mouse models constitutively expressing nitric oxide synthase will be useful in the study of these clinical conditions. Transgenic mice have been generated that express the murine inducible nitrix oxide synthase (iNOS) under the control of keratinocyte- and melanocyte-specific promoters. Three different DNA constructs were made to generate the transgenic mice. In the first construct, a 2-kb long mouse K14 promoter fragment was linked to the 3.9 kb mouse iNOS cDNA followed by a 2.1-kb hGH poly A sequence. In the second construct, a 3.7-kb involucrin promoter fragment was linked to mouse iNOS cDNA and a 0.2-kb SV40 poly A fragment. In the third construct, a 0.3-kb tyrosinase promoter fragment was linked to mouse iNOS cDNA and the SV40 poly A fragment. It is expected that the K14 and involucrin promoters would be active in keratinocytes while the tyrosinase promoter would be active in melanocytes. Fertilized B6C3F1 (C57B6/6 × C3H) mouse embryos were microinjected with the three constructs and transgenic founder mice (F0) were screened by PCR and Southern blots. F1 mice were generated by crossing littermates of different transgenes as well as with wild type B1/6C57 mice. Northern blot analysis and *in situ* hybridization revealed that some of the transgenic mice showed iNOS mRNA expression in skin. Several of the involucrin and tyrosinase transgenic mice exhibited various clinical and histologic phenotypes including loss of facial and abdominal hair consistent with alopecia areata, loss of hair color consistent with vitiligo, apparent thickening of the dermis suggestive of scleroderma, and a dermatitis. These results are consistent with the hypothesis that NO contributes to the pathogenesis of a number of skin conditions.

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Hair Morphogenesis Analysis of Follistatin-Deficient Mice and BMP-7 Knockout Mice

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Members of TGF-β/BMP family are involved in the control of hair follicle morphogenesis. The activity of several members of this family (activin, BMP-2, 4, 7) is usually antagonized by follistatin. The expression of BMP-7 has been reported in the inner root sheath cells and outer root sheath cells and the dermal papilla cells. Since follistatin-deficient mice show abnormalities in vibrissae follicles, we have further explored the role of follistatin and BMP-7 in hair follicle development with the analysis of knockout mice. Compared to wild type controls, follistatin knockout mice showed a significant retardation of hair follicle morphogenesis ($p < 0.01$). The follistatin null mutation also significantly reduced keratinocyte proliferation in stage 1-2 hair follicles. In contrast, BMP-7-deficient mice showed no significant abnormalities in hair follicle morphogenesis, keratinocyte proliferation, hair follicle density, or epidermal thickness. Immunohistological analysis of apoptotic cells (TUNEL), selected adhesion molecules (NCAM), morphogens (KGF), or growth factor receptors (FGFR-2, m-met) failed to reveal any substantial changes in both knockout strains. In organ culture of human anagen VI scalp hair follicles, recombinant human follistatin inhibited hair shaft elongation and induced hair follicle regression (catagen). Taken together, follistatin is involved in the control of both hair follicle morphogenesis and hair cycling. BMP-7 does not play an indispensable role in hair follicle development, possibly due to redundancy among various BMP family members.

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Androgens Modulate the Connective Tissue Phenotype of the Nipple

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The humoral hypercalcemia of malignancy factor parathyroid hormone-related protein (PTHrP) regulates a wide variety of developmental processes including tooth eruption, linear growth of long bones, branching morphogenesis of the mammary gland and patterning of the skin. Recently, we have demonstrated that keratin 14 (K14) promoter-mediated overexpression of PTHrP in the basal keratinocytes during development converts the entire murine ventral skin to hairless nipple sheath. At this point very little is known about the molecular basis for the morphologic differences between the nipple and surrounding skin or the role of sex steroid hormones in the patterning of this structure. In this study, we use electron microscopy, histochemistry, immunohistochemistry and image analysis to evaluate the murine nipple, as well as the ventral skin of male and female K14-PTHrP mice and ovarectomized and ocharectomized K14-PTHrP mice. The connective tissue of the nipple and the ventral dermis of female K14-PTHrP mouse have an increased number of cells, and is enriched in laminin, fibronectin and proteoglycans as compared to the ventral dermis of wild-type littermates. The increased cellularity of the nipple or nipple-like connective tissues was due in part to a significant increase in number/unit area of mast cells, capillaries and nerve fibers in the connective tissue. The adult male K14-PTHrP mouse also lacks hair on the ventral epidermis but does not manifest the nipple-like characteristics in the dermis. A developmental survey suggests the unique characteristics of nipple connective tissue appear 3 weeks after birth, suggesting a role for sex-specific steroid hormones in the final morphogenesis of this structure. Ovarectomy or ocharectomy had no impact on the dermal phenotype of the female or male K14-PTHrP mouse. Thus, it appears that the unique connective tissue phenotype of the mammalian nipple requires developmental expression of PTHrP but can be modulated by androgens.

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Lesions in Growth Factor Expression May Underlie Defects in Melanocyte Proliferation in Hair of Vitiligo Mice

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The coat color of vitiligo mice C57BL/6J-Mitfmi-vit, in which there is a spontaneous mutation at the mi(microphthalmia) locus, lightens progressively with aging. The change in coat color with aging in vitiligo mouse bears some resemblance to senile hair changes in humans. Immunohistochemistry and molecular biology tools were employed to investigate melanocyte changes, such as density and localization, in vitiligo mice as a function of age. For localization of melanocytes in hair, antibodies specific for tyrosinase-related protein-2, were employed. In the black hair of two month old vitiligo mice, elevated levels of follicular melanocytes were detected in the outer root sheath and in the hair bulb. A few scattered melanocytes were detected in the white hairs of vitiligo mice at 9 months of age. This decrease in numbers of melanocytes correlated with hair graying in vitiligo mouse. Using quantitative RT-PCR, we next examined gene expression of growth factors and their receptors associated with melanocyte proliferation. These included stem cell factor (SCF), Endothelin, nerve growth factor, hepatocyte growth factor, and basic fibroblast growth factor. Gene expression of 2, 6 and 11-month-old vitiligo mice were compared. Quantitative RT-PCR demonstrated that melanocyte gene expression was markedly decreased not only for growth factor receptors, but also for two paracrine factors, SCF and Endothelin. These results suggest that the mutation at the mi locus induce down regulation of gene expression not only for tyrosinase and tyrosinase-related protein-1 in melanocytes but also for growth factors associated with melanocyte proliferation, and their receptors.

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Unique Form of Murine Alopecia: A Mouse Model for Human Hair Disorders

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The rodent has been a useful model and biomedical tool for studying human hair disorders. An undocumented form of alopecia in CD^{-1} mice has been maintained over several years by selective breeding. Spontaneous alopecia occurs in 25–30% of the selected population and affects females 3 times more often than males. The process of hair loss normally begins after sexual maturity in both sexes. In affected mice, normal hair growth occurs until approximately 40 days of age and is subsequently followed by rapid hair loss (few days) from a rectangular area between the ears. This localized alopecia lasts for months and the bald patches appear to fill in a majority of mice following a variable period of time. Over the generations, some individuals became thinned rather than bald in the rectangular area. Histopathology from the established bald patches reveals an absence of matured hair follicles and exhibits no peri- or intrafollicular lymphocyte infiltration. Initial pedigree analysis suggests the involvement of two or more recessive alleles, with further genetic analysis necessary to elucidate the mode of transmission. This localized and rectangular pattern of murine alopecia may be a potentially useful model for the major types of human alopecias.

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Developmental and Breed-Related Changes in the Canine Hair Follicle

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The diversity of hair morphology and growth seen in different murine strains is approached, if not exceeded, only by the coat differences in canine breeds; however, little is known about hair growth in the dog. In this study we define breed- and age-related differences in the follicular morphology and hair cycle in 36 male dogs from three canine breeds (12 Siberian Huskies, 12 Labrador Retrievers and 12 Miniature Poodles). From 10 weeks to 81 weeks of age, the dogs were periodically sampled. The hair follicles were assessed using (1) optical-based fibre diameter and curvature analysis; and (2) vertically and horizontally sectioned 6 mm punch biopsy samples to define the morphology, number of hairs/compound follicle and anagen:telogen ratios. Evaluation of horizontal histologic sections confirmed the compound nature of the canine follicle. Compound follicles were present in groups we termed a follicular unit. Each compound follicle had the largest diameter follicles present anterior and the smallest posterior. In each breed there was an increase in the number of follicles until week 45. Siberian Huskies had the greatest number of follicles/follicular unit. Optical-based fibre diameter and curvature analysis identified Labrador Retrievers as the breed with the largest mean diameter of hairs and the least degree of curvature. Miniature Poodles had the smallest mean hair diameter throughout the study and the greatest curvature after week 54. As Labrador Retrievers and, to a lesser extent, Siberian Huskies matured (i.e. ''lost their puppy coat''), there was a transient production of coarser, straighter hairs. The trend in Siberian Huskies and Labrador Retrievers was an increase in the number of telogen follicles. By week 81, both breeds had approximately 70% of their hairs in telogen. For Miniature Poodles there was a decrease in the number of telogen follicles and by week 81 only 7% of the follicles were in telogen. These data indicate that the profound gross differences in the pelage of canine breeds can be defined morphologically and morphometrically. The breed differences, the unique orientation of the canine compound follicle (larger hairs anterior, smaller hairs posterior) and the recognition of breeds with anagen-predominant and telogen-predominant hair cycles suggest the dog hair follicle may be used to answer questions about hair growth not easily asked in murine or human systems.

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An Investigation into the Effects of Testosterone on Follicular Development Using a Novel *In Vitro* Culture Model of Antler Velvet

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The skin (velvet) covering growing deer antlers is an unique example of *de novo* development of new skin and hair follicles, i.e. ''neogenesis'' (a process directly equivalent to skin development in an embryo) occurring in an adult. Red deer velvet is being investigated as a novel model in which to study follicular embryogenesis. Immunohistological investigations have shown similar follicle development and characteristics to other mammalian systems. A particularly important feature of velvet is that several stages of follicular development are present in the same section due to the antlers very rapid growth. Since an *in vitro* system would be useful for investigating the roles of specific factors in embryogenesis, samples of antlers velvet have been cultured. As the antler is an androgen responsive organ the effects of testosterone on this system was also investigated. Skin biopsies (4 mm) from the tips of red deer (Cervus elaphus) antlers were cultured in DMEM in the presence or absence of 10 nM testosterone. Samples were supported in absorbable gelatine sponge (Gelofoam(r)). Samples were removed every 2 days up to day 7/8 and frozen. Frozen sections (6–7 μm) were stained with the histological stain SACPIC and antibodies to cytokeratin 6, the mesenchymal marker, vimentin and the cell proliferation marker, Ki67. The histology remained good until at least day 7 and under conditions with and without testosterone samples retained good morphology with Ki67 staining as normal at day 2 and still present at day 6. By day 6 cytokeratin 6 expression was found to be normal in the centre whereas at the biopsy periphery evidence of wound healing events was evident. Mesenchymal fibroblast aggregations stained for vimentin appeared normal at day 6. Results with, and without, testosterone were indistinguishable. These studies show that antler velvet can be cultured successfully and that testosterone does not have an appreciable affect on follicular development in antler velvet. Cultured antler skin should be a useful model system to investigate molecules involved in intercellular signalling in developing hair follicles without the ethical difficulties often associated with such research.

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Gene Therapy of Growing Hair Shafts

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A novel gene therapy technology of hair follicles has been developed which results for the first time in efficient genetic alteration of the hair shaft phenotype. Mouse anagen skin fragments in histoculture were transduced at high efficiency by adenoviral-green fluorescent protein (GFP) with subsequent grafting of the skin fragments to nude mice. The histocultured skin fragments were treated with collagenase which made hair follicles accessible to the adenoviral-GFP gene allowing high-efficiency transduction. Without collagenase treatment, GFP expression was negligible. The extent of GFP expression in the hair follicle depended on adenoviral titer and time of adenoviral treatment. After transplantation of the GFP-transduced skin to nude mice, GFP was readily visualized in as many as 75% of hair follicles including large numbers of GFP-fluorescent growing hair shafts. High-level transgene expression was maintained *in vivo* in hair follicles sufficiently such that growing hair shafts were phenotypically altered as demonstrated by specific GFP fluorescence in the hair shaft. RT-PCR measurement of GFP-gene expression confirmed GFP-gene transfer and expression in the hair follicle. This novel technology demonstrates the feasibility of efficient genetic modification of the hair shaft.

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Gender Difference in Autoimmune Alopecia of New Zealand Black/KN Mouse

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New Zealand Black (NZB)/KN mouse is an SLE-prone mouse associated with arthritis, and male mouse shows alopecia spontaneously. We have reported that the male NZB/KN mouse is an autoimmune alopecia model, which suggests that the gender is an important factor influencing the development of alopecia. To verify the male predominance, we studied the relationship among alopecia, gender, and other clinical symptoms using NZB/KN, the F1 hybrid mouse with NZ White(NZW) mouse and the F2 hybrid mouse of (W/BKN F1Â~W/BKN F1). The incidence of alopecia of male NZB/KN, W/BKN F1, B/WKN F1, and F2 mice at 12 months (mo) were 88%, 9.5%, 0%, and 44%, respectively. Male NZB/KN mouse initially developed hair loss and a slight indurative change around the tail, and then on the upper back. Hair density at alopecia lesion of male NZB/KN mouse was lower than it's peripheral skin and control skin, although there was no difference about diameter. Histologically, intrafollicular lymphocyte infiltration were seen at the primary lesion and intradermal perivascular mononuclear cell infiltration were observed in the fully developed lesion. In the serum mainly Ig M type antibodies against follicular structure were detected at 3 month, and 75% of male NZB/KN showed the positive titer until 12 month. Immunofluorescence study revealed IgM deposition at basement membrane zone of both epidermis and/or hair follicle. In female NZB/KN mice a slight hair loss was found in 24% on the upper back at 12 month, but the lesion was not fully developed. Female W/BKN F1, B/WKN F1, and F2 mice showed alopecia in 6%, 0%, and 7.3%, respectively. F2 mice showed significant correlation among gender, alopecia and proteinuria, but not among alopecia, H-2, and splenomegaly. The alopecia in male NZB/KN mouse is a novel model of autoimmune alopecia and found to be closely associated with male gender based on the studies on F1 and F2 hybrid mice.

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Restoration of Hair Growth with Topical Anthralin in Alopecia Areata Rats: Efficacy and Molecular Mechanisms

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Anthralin has been suggested to have potential efficacy in alopecia areata (AA). It may have a nonspecific immunomodulating effect on AA as it does in the topical treatment of psoriasis. The aim of the present study was to investigate the therapeutic effects of anthralin on AA-affected rats and the underlying mechanisms at the molecular levels. The Dundee experimental bald rats (DEBR) were treated daily for 11 weeks on half of the dorsal skin with 0.1% anthralin ointment and the contra-lateral sides were treated with the vehicle ointment. All 15 rats showed near complete hair regrowth on the treated sides, while the control sides remained balding. At the end of the treatment, skin biopsies were collected from both treated and control sides. RNA and proteins were extracted. Proteins were used for KinetworkTM analytical screens for cell signaling proteins (Kinexus, Canada) to determine the expression of various protein kinases and kinase activities which might be responsible for the signal transduction mediated by anthralin in rat skins. RNA protection assay was performed to determine the gene expression of various cytokines mediated by anthralin. Among the cytokines we have tested by RPA, IL-1RA and INF-γ showed remarkable reduction after anthralin treatment. We conclude that anthralin is 100% effective on restoring hair in DEBR rats with AA-like hair loss. The molecular mechanism underlying the efficacy of anthralin on hair regrowth in AA rats might be mediated by the interplay of cytokines and protein kinases produced locally in the skin upon anthralin treatment.

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Genetic Analysis of the Interleukin-1 Receptor Antagonist and its Homologue IL1-L1 in Alopecia Areata: Strong Disease Associations and Possible Gene Interaction

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Alopecia areata is an inflammatory hair loss disease with a major genetic component. The presence of focal inflammatory lesions with perifollicular T-cell infiltrates reflects the importance of local cytokine production in the pathogenesis. In addition to its fundamental pro-inflammatory role, the IL-1 system has major effects on hair growth regulation *in vitro*, with the inhibitory actions of IL-1α and IL-1β being opposed by the receptor antagonist, IL-1ra. The novel interleukin-1 like molecule 1 (IL-1L1) which has greatest gene sequence homology with *IL1RN*, the gene encoding IL-1ra, is another potential IL-1 antagonist. In view of previous studies suggesting a significant role for *IL1RN* polymorphisms in the pathogenesis of autoimmune/inflammatory disease, we have analysed polymorphisms of IL-1ra (*IL1RN* + 2018) and its homologue IL-1L1 (*IL1L1* + 4734) in a case–control association study on 165 patients and a large number of matched controls. Homozygosity for the rare allele of *IL1RN* (*IL1RN**2) was significantly associated with alopecia areata [odds ratio (OR) = 1.89, 95% CI (1.09,3.28); p = 0.02] confirming our previous findings of significant association with the *IL1RN* VNTR. The results also revealed a novel association involving a polymorphism of the interleukin-1 receptor antagonist homologue *IL1L1* at position + 4734, *IL1RN* + 2018 and alopecia areata. Genotypes combining at least 3 copies of the rare alleles for *IL1RN* & *IL1L1* conferred a more than additive increase in the risk of disease compared to *IL1RN* + 2018 or *IL1L1* + 4734 alone [OR 3.37 (1.60, 7.06); p = 0.002], suggesting possible synergy between *IL1RN* and *IL1L1*. This effect was stronger in patients with severe disease (alopecia totalis/universalis) [OR 4.62 (1.87, 11.40), p = 0.0022], and in those with early age at onset (< 20 year.) [OR = 6.38 (2.64, 15.42), p = 0.0002]. Our results suggest that these polymorphisms of *IL1RN* & *IL1L1* themselves or a gene in linkage disequilibrium with *IL1RN* & *IL1L1* predispose to the more severe forms of alopecia areata.

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Human Gene Expression Mapping in Alopecia Areata

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Alopecia areata (AA) is a disease characterized by the loss of hair that affects approximately 2–4 million individuals in the US, including men, women, and children. Numerous studies have suggested a genetic predisposition to AA and increasing experimental evidence suggests that AA is autoimmune in nature. However, mechanisms of disease initiation and development are not fully understood. Although the expression levels of several genes have been shown to be altered in scalp tissue affected by AA, a broad perspective on the gene expression abnormalities characterizing AA has been unavailable. In this study, we have used microarray analysis to determine the gene expression differences between normal scalp and AA affected scalp at a genome-wide scale. Total RNA was prepared from lesional and site-matched nonlesional scalp biopsy samples from 5 patients with alopecia areata. The total RNA was enriched for mRNA, and cDNA was then synthesized, radiolabeled, and hybridized to Atlas Human Cytokine/Receptor Arrays. These arrays allow the simultaneous analysis of expression of 265 genes. Expression was quantitated using a Storm 840 PhosphorImager and analysis was performed using ImageQuant 1.2 software (Molecular Dynamics). Preliminary results show that approximately 20 genes exhibited differential expression, defined as a two-fold or greater difference in expression between lesional and nonlesional tissue. Differentially expressed genes include the interleukin-1 receptor antagonist protein precursor, the hepatocyte growth factor agonist, the keratinocyte growth factor, the interleukin-6 receptor α subunit, and the interferon-γ receptor. We are currently confirming these results on a larger number of samples using Affymetrix Human Genome U95A Arrays, which permit the simultaneous analysis of expression of approximately 12 500 sequences. We expect these studies to enhance our understanding of basic disease mechanisms and illuminate potential therapeutic targets.

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Melanocyte Associated Antigens can Serve as Autoantigens for Alopecia Areata

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Alopecia areata (AA) is an tissue restricted autoimmune disease affecting the hair follicle, resulting in hair loss. It has been hypothesized that the autoantigens of alopecia areata are melanocyte associated. The goal of this study was to determine if melanocyte associated antigens can activate T-cells from alopecia areata patients to induce hair loss. The role of CD8+ T-cells in transferring hair loss directed the search to HLA-A2 restricted melanocyte epitopes. Potential autoantigens were tested in the human scalp explant/Prkdc(SCID) mouse transfer system. Human, and animal protocols were approved by the appropriate institutional committees. Scalp T-cells from lesional AA scalp, were cultured with APC and a source of potential antigen, along with IL2. The T-cells were then injected into autologous scalp grafts, and hair loss measured. Hair follicle homogenate was used as a positive control for inducing hair loss. (1) In the initial experiment with 4 AA patients, human melanoma homogenate (MeWo cells) was used as a source of potential antigen. T-cells cultured with melanoma homogenate induced statistically significant reduction in hair growth ($p < 0.01$ by ANOVA). (2) HLA-A2 restricted melanocyte T-cell epitopes were then tested with 6 HLA-A2 positive AA patients. PBMC proliferation was used as a guide for selecting peptides for scalp T-cell stimulation. Peptide activated T-cells reduced the number of hairs re-growing in 2 experiments with 6 patients (Exp 2: $p < 0.001$ by ANOVA). The most consistent peptide autoantigens in this series were the Gp100 derived G9-209, and G9-280 peptides, as well as MART-1 (27-35). Melanocyte peptide epitopes can function as autoantigens for AA. Multiple peptides were recognized, suggesting epitope spreading.

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Can Alopecia Areata-Like Hair Loss be Transferred to Naïve Mice with IgG from Alopecia Areata-Affected Animals?

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Alopecia areata (AA) is a presumptive autoimmune hair loss disorder affecting humans and several other mammalian species. Previously we have demonstrated the presence of antihair follicle (HF) auto-antibodies in humans, horses and dogs with AA as well as in the C3H/HeJ AA mouse model. However, it is unclear whether these antibodies are pathogenic. Some suggest they result secondary to follicular damage, although anti-HF antibodies can appear prior to the onset of hair loss. Recently, we reported limited telogen arrest and shedding in murine skin after passive transfer of equine AA immunoglobulin and now extend this investigation by passively transferring purified IgG from a new case of equine AA into murine haired skin. The serum used for this study were selected on the basis of high titer anti-HF antibodies by indirect immunofluorescence and Western blotting with reactivity re-tested after IgG purification. Eight C57Bl/10 mice in anagen III/IV were injected intradermally with 5 mg IgG twice weekly for 44 days. Thereafter, dorsum skin was harvested at both 7 h and 10 d, 3 mths and 5 mths post final injection for H&E and TEM. Full hair regrowth was observed around the injection site in all mice by 12 days post first injection. However, the injection site itself remained hairless for a further 14 days in mice treated with normal IgG but did not regrow until 57 days later in mice treated with AA IgG. The AA IgG-treated peri-injection site skin revealed a spatially restricted catagen-precipitation of hair follicles that was associated histologically with the massive mixed-cell peri/intrafollicular and dermal inflammatory infiltrate. Furthermore, the affected hair follicles exhibited a telogen arrest while distant follicles continued to cycle normally. Other notable findings included epidermal hyperplasia at the AA IgG-treated site. None of these features was observed in normal IgG-treated skin. Isolated areas of pigmentation loss were observed in both AA-treated and control IgG-treated mice. While this study suggests that AA IgG injected into murine haired-skin can have local effects on hair cycling consistent with an inflammatory response; a pathogenic role for antihair follicle antibodies in AA awaits confirmation.

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The Role of Fas and Fas Ligand in Alopecia Areata of C3H/HeJ Mice

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Interaction of Fas ligand (FasL) with its receptor Fas leads to apoptosis of the Fas-expressing cell. Fas has been shown to be expressed on hair follicles and FasL on cells of perifollicular infiltrates in alopecia areata (AA) patients. Fas-deficient Fas^{lpr}/Fas^{lpr} and Fas ligand-deficient *Faslgld/Faslgld* mice are relatively resistant to induction of T-cell mediated autoimmune diseases. Spontaneous AA develops in 20% of C3H/HeJ mice, but grafting AA affected skin to normal haired C3H/HeJ mice or mice from other histocompatible strains will induce the disease in graft recipients. Because AA is regarded as a T-cell mediated autoimmune disease, we addressed the question whether Fas^{lpr}/Fas^{lpr} and *Faslgld/Faslgld* mice are resistant to the induction of AA. Lesional AA skin was grafted onto C3H.MRL-Fas^{lpr} mice, C3H/HeJ-$Fasl^{gld}$ mice and control C3H/HeJ+/+ mice. Control mice developed AA within 10 weeks, whereas C3H.MRL-Fas^{lpr} and C3H/HeJ-$Fasl^{gld}$ mice did not develop AA within 20 weeks after grafting. Immunohistochemistry revealed a peri- and intrafollicular lymphocytic infiltrate of $CD4^+$ and $CD8^+$ T-cells around anagen hair follicles and an aberrant expression of MHC class I and II on hair follicle epithelium in control mice. In contrast, C3H.MRL-Fas^{lpr} and C3H/HeJ-$Fasl^{gld}$ had no perifollicular infiltrate and no aberrant expression of MHC class I and II. Immunofluorescence staining revealed an increased expression of Fas on hair follicles in control mice with AA while Fas expression was absent in C3H.MRL – Fas^{lpr}mice. TUNEL staining showed an increased number of apoptotic cells in control, AA affected mouse hair follicle eptihelium, whereas in C3H.MRL-Fas^{lpr} and C3H/HeJ-$Fasl^{gld}$ mice there were almost no apoptotic cells in hair follicles. Our results show that C3H.MRL-Fas^{lpr} and C3H/HeJ-$Fasl^{gld}$ mice are resistant to induction of AA. These findings suggest an important pathogenetic role of the Fas-Fas ligand system in AA.

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A Family Case of Alopecia Universalis Congenita is Not Linked to Hairless Gene

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Inheritied forms of hair loss without associated skin or internal abnormalities are rare diseases. Two autosomal recessive types of this condition have been described: alopecia universalis congenita and atrichia with papular lesions. The two diseases have been shown to results from a mutation in the human hairless gene. We report a Japanese family of alopecia universalis congenita with autosomal dominant mode of inheritance, in which a 28-year-old father and his two sons were affected. No associated ectodermal defects were noted. In mutation analysis for the father, no mutation was identified in hairless gene. These results suggest that there may be another gene responsible for alopecia universalis congenita.

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Recapitulation of the Hairless Mouse Phenotype using Catalytic Oligonucleotides

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Ribozyme technology is widely used to target mRNA in a sequence specific fashion, and thus change the expression pattern of cells or tissues. While the goal of mRNA targeting is usually the cleavage of mutant mRNA with the prospect of gene therapy for inherited diseases, in certain instances targeting of wild-type genes can be used therapeutically. Lack of expression of the mouse hairless gene due to inherited mutations leads to the complete loss of hair, known as atrichia. We designed this study to recapitulate the hairless phenotype in a restricted manner by topical application of deoxy-ribozyme targeting molecules to specifically cleave the mouse hairless mRNA. The targeting oligonucleotides were delivered on the back of C57Bl/6 J mice using a commercially available liposome reagent. The delivery began immediately after birth, and was repeated daily until day 16. Samples for pathology were taken at days 22 and 35, processed and stained using standard techniques. The pathology samples of the treated area from day 22 demonstrated a decreased number of hair follicles, involution of the remaining follicles and separation of the dermal papillae, all characteristic of the hairless phenotype. In contrast, the pathology samples from the nontreated area of the same animal did not show these alterations. The pathology samples of the treated area from day 35 demonstrated the presence of large dermal cysts characteristic of the hairless phenotype, but not normally present in the skin of C57Bl/6 J mice. In this study, we successfully recapitulated the hairless phenotype using topically applied target specific catalytic oligonucleotides designed to cleave the mouse hairless mRNA. Our results demonstrate the feasibility of using ribozyme technology to alter gene expression in the skin via topical application, and provide proof of principle for the development of this strategy for permanent hair removal.

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Atrichia Caused by Mutations in the Vitamin D Receptor Gene is a Phenocopy of Generalized Atrichia Caused by Mutations in the Hairless Gene

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Generalized atrichia with papules is a rare disorder characterized by loss of hair shortly after birth and development of cutaneous cysts. Mutations in the hairless gene (HR) cause this phenotype in both mouse and human. Here we present a case of atrichia with papules in a patient with a normal HR gene, but with mutations in both alleles of the VITAMIN D RECEPTOR (VDR). The patient exhibited vitamin D resistant rickets, which was confirmed by an absent response of her fibroblasts to 1,25 dihydroxyvitamin D3 *in vitro*. Strikingly similar to individuals with HR mutations, her skin showed an absence of normal hair follicles and the presence of cysts and follicular remnants containing keratin-15 positive cells suggesting epithelial stem cells are still present. Although hair loss has been reported in association with hereditary vitamin D resistant rickets (HVDRR), we now characterize this alopecia as clinically and pathologically indistinguishable from generalized atrichia with papules, which was previously thought to be caused only by mutations in HR. These findings suggest that VDR and HR, which are both zinc finger proteins, may interact and control postnatal cycling of the hair follicle.

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Atrichia with Papular Lesions Resulting from Compound Heterozygous Mutations in the Hairless Gene: A Lesson for Differential Diagnosis of Alopecia Universalis

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Atrichia with papular lesions (APL) is a rare, autosomal recessive form of total alopecia, in which mutations in the hairless (h) gene have been shown to underlie the phenotype. We suspect that APL is actually much more common that previous population estimates suggest, because we believe the disorder is frequently misdiagnosed as alopecia universalis. To date, mutations identified in the hairless gene have all been homozygous mutations in probands within families with extensive consanguinity, where identity-by-descent (IBD) was anticipated. We sought to investigate whether APL might also be found among patients in small families without obvious IBD, particularly those giving a history of (1) normal hair at birth which was shed and never regrew; and (2) ''alopecia universalis'' that is recalcitrant to any treatment. In this study, we identified a small family of German origin in which two of four siblings were affected and gave this clinical history. Direct sequence analysis of the h gene in one proband revealed distinct mutations on each allele. The first was a 2-bp deletion of the splice acceptor site of intron 13 (2847–2delAG), and the second was a nonsense mutation in exon 19 (Q1176X). This is the first demonstration of compound heterozygous mutations in the h gene. These findings support the hypothesis that APL can exist in small families without IBD, and underscore the possibility that APL may be masquerading clinically as alopecia universalis. We anticipate that increased recognition of this disorder will result in accurate discrimination between APL and alopecia universalis, thus sparing unnecessary treatment to patients affected with APL.

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Clinical and Molecular Diagnostic Criteria of Congenital Atrichia with Papular Lesions

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Congenital atrichia with papular lesions is a rare, autosomal recessive form of total alopecia and mutations in the hairless (h) gene have been implicated in this disorder. Published estimates of the prevalence of this disorder remain surprisingly low considering pathogenetic mutations in h have been found in distinct ethnicities around the world. Therefore, it is likely that congenital atrichia with papular lesions is far more common than previously thought and often mistaken for its phenocopy, the putative autoimmune form of alopecia universalis. To clarify this discrepancy, we propose criteria for the clinical diagnosis of congenital atrichia with papular lesions. Among these is the novel report of the consistent observation of hypopigmented whitish streaks on the scalp surface of affected individuals. Additionally, we report the identification of a novel missense mutation in h from a family of Arab Palestinian origin that exhibit the pathognomonic symptoms of atrichia with papular lesions. Collectively, we anticipate that an increased recognition of this disorder will result in more accurate diagnosis and the sparing of unnecessary treatment to patients.

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Computer-Assisted Histomorphometric Evaluation of Scalp Biopsies

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A major challenge in the histophathologic examination of scalp biopsies is to adequately visualize and evaluate as many of the hair follicles present in the tissue. While routine vertical sections are easier to process and exhibit familiar histological landscape, oblique orientation of the follicular structures precludes any means of quantitation even if multiple serial vertical sections are available. To improve on the diagnostic yield of conventional vertical sections, the use of transverse sections of a scalp punch has been suggested. The demonstration of all the follicular structures simultaneously in one transverse tissue section allows morphometric analysis of parameters such as hair density and diameter. An obvious disadvantage of transverse sectioning is that a very limited cross-section of each follicle is shown in each microscopic section and numerous sections are needed to examine the follicles in their entirety and to determine follicular growth activity. In the management of patients with alopecia, accurate morphometric analysis of hair growth is required. Recent advances in PC-based computing have enabled efficient processing of large number of images and this technology is well suited for the study of transverse sections of scalp biopsies. To ensure complete coverage of all the follicular structures, scalp biopsies from 12 patients with noninflamatory, nonscarring alopecia have been serially sectioned in 0.1 mm interval, resulting in 30–50 sections in average for each case. Imaging software including Align(r), Trace(r) and 3D exploration(r) are then used to evaluate and quantify the follicular structures, and the results are presented in a 3 dimensional graph format using Excel(r). In addition a computer generated 3 dimensional model of each biopsy tissue core is generated, allowing easy visualization of all hair structures. Out data suggests that the anatomy of follicular structures and scalp hairs, including density and diameter, is more variable than previously reported. Up to 55 hairs have been counted in a 4.0-mm punch and our 3D models demonstrate the occasional merging of follicular infundibulae and existence of dystrophic follicular units. Patients with nonscarring, noninflammatory alopecia may suffer from overlap of diseases which can only be revealed by detailed histomorphometric studies. We believe computer assisted analysis of the scalp biopsy is the best means to further elaborate variations of human scalp hair and to investigate cases of alopecia.

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TrichoScan: Combining Epiluminescence Microscopy with Digital Image Analysis for the Measurement of Hair Growth *In Vivo*

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Numerous methods have been reported to assess the rate of hair growth, but most techniques are of little use to the clinician, because they are time consuming, costly or difficult to perform. Therefore, an operator- and patient-friendly, inexpensive, validated and reliable method is a rational need. Such a method must be able to analyze the biological parameters that constitute hair growth, which are: 1: hair density (n per cm^2), 2: hair diameter (μm), 3: hair growth rate (mm per day) and 4: anagen/telogen ratio. We have developed such a method which combines an epiluminescence microscopy with a newly programmed digital image analysis. The application of the technique is demonstrated in 30 volunteers (with or without AGA) where a scalp area of 1.8 cm^2 was clipped, dyed and landmarked with a central, single black tattoo. Images were taken at 20 and 40-fold magnification immediately and 2 days after shaving, in three monthly periods. Our results show that the technique is able to automatically analyze the anagen/telogen ratio (<18% telogen hairs in scalp unaffected by AGA) and hair growth rate (mean 0.31 mm per day). We were able to demonstrate that within the very small analyzed area (0.225 cm^2) the total hair number and hair thickness at the occiput did not change during 6 months, but we observed a decrease in hair number (–0.43 hairs) and total thickness (–1.85 μm) in AGA-affected untreated scalp in contrast to an increase in hair number (+4.7 hairs; $p = 0.059$) and total hair thickness (+4.85 μm; $p = 0.039$) after six months of treatment with finasteride. The advantage of this technique is that it can be used for clinical studies to compare placebo versus treatment, or to compare different capacities of different hair-growth-promoting substances. This technique can be used for studying AGA or other forms of alopecia, but in addition can be adopted to study the effect of drugs or laser treatment on hypertrichosis or hirsutism.

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Eflornithine Hydrochloride Cream 13.9% Disrupts Actin Filaments of the Beard Hair Follicle in Men of African-American Descent with Pseudofolliculitis Barbae (PFB)

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The purpose of this study was to obtain information on the mechanism of action of eflornithine hydrochloride cream 13.9% in the treatment of Grade 3 PFB in adult males of African-American descent. In cultured keratinocytes, treatment with a-difluoromethionine (eflornithine hydrochloride) not only inhibits ornithine decarboxylase (ODC), but also disrupts cytoplasmic actin organization and ODC localization. We hypothesized b.i.d. application of the cream for 16 weeks to the beard region of 10 men would alter the cytoskeleton of the beard hair follicle and translate into altered hair follicle growth and integrity. Two 4-mm punch biopsy specimens were obtained pre- and post-treatment from lesions in the beard region. Biopsy samples were fixed in Zamboni's fixative for 24 h at room temperature or 4°C and later stored in cryopreservative at 4°C until stained. Biopsies were cut into 400-micron vertical sections and stained as floating sections with multiple labels including phalloidin, conjugated to the red fluorophore Texas Red, that labels actin bundles/stress fibers. Fluorescent-stained sections were analyzed using laser scanning confocal microscopy (LSCM). At the end of the treatment course, clinical severity of PFB improved in the majority of patients, the number of miniaturized hair follicles increased and the number of large curved follicles decreased. LSCM analysis revealed changes in actin filament architecture in the keratogenous zone of the outer root sheath of anagen beard hair follicles. The keratogenous zone is an area above the hair bulb through which enlarged hair matrix cells are funneled, resulting in condensation and elongation of these cells. After treatment the actin filament bundles appeared very disorganized and lost their parallel orientation. Actin filament bundles of anagen follicles are analogous to stress fibers of nonmuscle cells, such as red blood cells, providing mechanical support, cell adhesion, and contractile functions. We suggest that eflornithine hydrochloride cream 13.9% disrupts the actin cytoskeleton of the actively growing beard hair follicle. Disruption of this scaffolding could lead to subsequent loss of follicle integrity and alteration of growth characteristics of the beard hair follicle.

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Inhibition of Ornithine Decarboxylase: Effect on the Hair Follicle and Epidermis of Patients with Pseudofolliculitis Barbae (PFB) Treated with Eflornithine Hydrochloride Cream 13.9%

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Eflornithine hydrochloride is an irreversible inhibitor of the enzyme ornithine decarboxylase. In this study, we examined the effect of b.i.d. application of eflornithine hydrochloride cream 13.9% on the hair follicle, hair fiber, and epidermis of men with PFB. Our hypothesis was that alterations in epidermal thickness, hair follicle structure and fiber would occur following therapy. Thirteen participants with at least a two year history of PFB, who shaved at least once weekly and who had a PFB severity rating of at least Grade 3 were enrolled; 10 completed the study which consisted of a 2-week pretreatment phase followed by visits at Days 0, 2, 7–10, 28, 56, 84, 112, 114, 121–124, and 140. Photographs, beard hair samples and 4-mm skin biopsies were obtained from PFB lesions at Days 2 and 114. Beard hair fibers were nonspecifically stained with Alexa Fluor(r) 594 C5 maleimide, sodium salt from Molecular Probes. Cuticular, cortical and medullary staining was examined using conventional epifluorescence microscopy. Biopsy samples were fixed in 10% buffered formalin, embedded, cut into 7 micron sections and stained with either hematoxylin and eosin or antibody to Ki67. This antibody binds to nuclear antigen(s) and is associated with cell proliferation throughout the cell cycle. The number and type of follicles were counted, samples were examined for changes in inflammation, epidermal thickness and differentiation. No significant differences in cuticular, cortical, or medullary staining was seen. In the post-treatment samples (1) miniaturized follicles were noted in 6 of the 10 biopsy samples, (2) epidermal thickness remained the same in all samples and (3) epidermal differentiation appeared more organized. Examination of Ki-67 staining in the epidermis revealed a decrease in epidermal staining in all but 2 patients. These observations are consistent with our knowledge that eflornithine hydrochloride is an irreversible inhibitor of ornithine decarboxylase and should be an effective drug to use in the management of PFB and possibly other skin diseases characterized by abnormal cell proliferation.

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Soymilk Reduces Hair Growth and Hair Follicle Dimensions

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Soybean extracts and the soybean-derived serine protease inhibitors STI and BBI were shown to reduce skin pigmentation, suggesting that soymilk could be used as a natural alternative to skin lightening. To examine the possible effect of STI, BBI and soymilk on hair pigmentation we used the mouse induced hair cycle model system. C57Bl/6 and C3H mice, age 9 weeks, were wax depilated to induce a new hair cycle, and were treated daily with soymilk, STI and BBI. Interestingly, we found that these agents affect not only hair pigmentation, but also the rate of hair growth, the dimensions of the hair follicle and hair shaft, and the appearance of the hair. For the first time it is demonstrated both morphological and histologically that soymilk and the soybean-derived serine protease inhibitors could be used to prevent unwanted hair growth. These agents could reduce the rate of hair growth, decrease hair shaft dimensions and alter the pattern of melanogenic gene expression.

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The Vertical Hair Density in Japanese Male with Androgenetic Alopecia is not Significantly Different from that in Nonbald Male

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Androgenetic alopecia (AGA) is the most common type of baldness with vellus hairs and completely atrophic follicles. The duration of hair cycle of vellus hair is shorter than that of terminal hair. The balding process is usually gradual the miniaturization of hair follicle (vellus hair) and successive hair shading. However the contributions of population of miniaturized or lost hairs on the AGA progressing are still unclear. We investigate the hair density and hair diameter in Japanese male with or without AGA to discuss the relationships between these data and overall hair appearance. The subjects between 15 and 62-year-old with (n = 258) or without (n = 123) AGA were studied. The subjects with AGA classified in classes III-vertex, IV, V or VI on the Hamilton-Norwood classification. The inspected alopecia scores were evaluated on the top view photographs according to compare with the prevalidated reference scale from 1 (slightly bald) to 10 (bald). The vertex hairs in areas of 5×5 mm were cut with scissors to record the images by video microscope. The hair cut area and the number of cut hairs calculated as the pixel numbers and counted on the images, respectively. The hair diameter also calculated as the pixel numbers on magnified images. The hair density distributed approximately from 125 to 325 cm-2 in both the nonbald and bald subjects. The average of density in bald subjects is not significantly different from that on nonbald subjects. In contrast the average of hair diameter correlate with the alopecia scores. It is concluded that the appearance of hair loss on the AGA progressing at least until the class VI on the Hamilton-Norwood classification results mainly from the miniaturization of hair follicle, but not from the hair shading. We therefore believe that the prolongation of anagen duration is important to improve AGA.

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Reversibility of Hair Follicle Changes Before 30 Months of Androgenetic Alopecia

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We studied horizontal and vertical biopsy from 15 caucasian 24–41-year-old males diagnosed with bitemporal recession Androgenetic Alopecia (AA) for 1.5–18 years (average 7.4 years). All 15 biopsies were stained with H&E, Van Gieson and with other phenol specific stainings. We found (1) decreased number of hair follicles (HF) 1.75–2.45 per sq. mm from 3.5 to 5 per sq. mm in control group, (2) infundibulum of HF dilatated 124–192 mm and most of them covered with keratinazed plug lacking normal hair shaft growth. (3) Eleven pts with AA longer than 3 years had severe perifollicular fibrosis – collagen fibers were compact and formed a small scar-like formation around each anagen hair follicle. Two patients – 33-year-old with 18 month AA and 23-year-old with 20 month AA did not have these hair follicle changes. Two 26-year-old patients with 30 and 36 month AA, respectively, were found to have some not so severe collagen fiber changes. (4) None of Anagen HF was situated in subcutaneous fat. The result of this study is that any available treatment of AA is recommended to start earlier than 30 months from first signs of AA, to avoid irreversible collagen changes associated with loss of normal blood supply, innervation, etc. And leading to miniaturization and preventing hair from normal cycling.

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17α-Estradiol Induces Aromatase Activity in Isolated Human Hair Follicles

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Women tend to develop AGA later and milder than men, but with the decline of serum estrogens during the menopause many women show an accelerated progression of AGA. Estrogens may play a protective role against the development of AGA, because pregnant women are in some way protected form androgenetic hair loss, but lose their hairs again postpartum. In Europe, topically applied estrogens such as 17β-estadiole are used to treat androgenetic alopecia, both in women and men. The femal hormone 17β-Estradiol can be used only in women, whereas the hormonally almost inactive isomer 17α-estradiol can be used in men as well. Although some clinical studies show considerable success of such an approach, the underlying pathways of 17α-estradiol-induced hair regrowth are unknown. It is likely not a receptor mediated hormon effect, since 17β-Estradiol is an hormon and 17α-estradiol not. Recently it has been shown that hair follicles from women with AGA express more aromatase activity compared to male hair follices, and interestingly those women taking aromatase inhibitors tend to develop rapidly progressive AGA. These circumstantial lines of evidence indicate a role of aromatase during the pathogenesis of AGA. In order to unravel the pathways of 17α-estradiol-mediated effcets on the hair follicles, we measured aromatase activity in isolated intact human occipital hair follicles by incubating hair follicles with H3–1β-androstenedione with or without 17α-estradiol (1 nM, 100 nM, 1 μM) for 24 or 48 h. In comparison to the controls (female, 444 fmol per mm^3 = 100%), we noticed a concentration- and time-dependent increase of aromatase activity in 17α-estradiol-incubated female hair follicles (e.g. 24 h: 1 nM = +18%, 100 nM = +25%, 1 μM = +57%; 24 h: 1 nM = +18%, 48 h: 1 nM = +25%). Our *ex vivo* results suggest that under the influence of 17α-estradiol an increased conversion of testosterone to 17β-estradiol and androstendione to estrone takes place. In theory this pathway may diminish the amount of intrafollicular testosterone available for conversion to DHT, and because DHT is the major mediator of AGA, this pathway may explain the beneficial effect of 17α-estradiol on the development and progression of AGA.

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Prevalence of Hair Disorders in the Women's Interagency HIV Study (WIHS)

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Hair disorders that have been reported in HIV-infected patients include diffuse hair loss, changes in hair texture, and trichomegaly. The prevalence of these disorders is unknown. Our objective was to determine the prevalence of hair disorders in a cohort of HIV-infected women and a comparison cohort of HIV-uninfected women. We analyzed a questionnaire administered to women enrolled in the Northern California Women's Interagency HIV Study (WIHS). Questions were asked about personal and family history of hair loss, current amount of hair, changes in hair texture, changes in eyebrow or eyelashes length, and hair grooming practices. 196 HIV-infected women and 50 HIV-uninfected women completed the questionnaire. In bivariate analyses, HIV-infected women were more likely to report changes in hair texture (47%) as compared to HIV-uninfected women (22%) ($p < 0.05$). 23% of the HIV-infected women reported thinner hair, 12% coarser, 6% curlier, 2% dryer and 1% straighter. Multivariate logistic regression showed both HIV-infection and older age to be independent predictors of change in hair texture (OR 3.1 CI 1.5–6.5 and OR 1.5 CI 1.1–2.2, respectively) Race was not associated with hair texture change. Five HIV-infected patients compared to no HIV-uninfected women reported longer eyelashes. Older age (but not HIV status) was associated with reported decreased hair density (OR 1.5 CI 1.1–2.2) There were no significant differences in family history of hair loss, eyebrow length, or hair grooming. In conclusion, the prevalence of hair texture change in this cohort of HIV-infected women is high (47%) and is significantly greater than in the comparison cohort of HIV-uninfected women (22%) ($p < 0.05$). Reports of longer eyelashes were exclusive to the HIV-infected women. Decreased hair density was associated with increased age but not HIV status in this cohort. Possible etiologies of hair texture change in HIV-infection may include HIV-viremia or medication use; further investigation is warranted.

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Histology and Hormonal Activity in Senescent Thinning in Males

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Senescent thinning of the scalp hair, or thinning that occurs after age 60, is poorly understood, and it is unclear if it is a distinct entity or a continuum of androgenetic alopecia (AGA). In a previous study, males age 18–30 with AGA had higher levels of 5α-reductase type 1 and 2, more androgen receptors, and lower levels of cytochrome p-450 aromatase in frontal hair follicles than in occipital follicles. This study in males age 60 years and older was designed to determine the histology and hormonal findings in older males with hair thinning. Males who experienced the onset of scalp hair thinning after age 60 were compared to age-matched males (controls) without hair thinning. Four scalp biopsies, two from the frontal and two from the occipital scalp, were obtained for horizontal sections and biochemical assay. Histologic findings were primarily follicular downsizing. Follicular drop out was not detected using elastic tissue stains; there was no significant difference in follicle number in frontal compared to occipital scalp. Senescent thinning was indistinguishable from androgenetic alopecia in older males. Inflammatory changes were not a significant feature. Biochemical analysis for androgen receptors, 5α-reductase type 1 and 2, and aromatase, in scalp biopsies from older males showed nearly a two fold decrease in levels compared to young males with AGA. In males over 60, androgen receptor and aromatase levels were low and comparable in frontal and occipital scalp regions with and without thinning. The 5α-reductase type 1 and 2 levels were only slightly higher in males with thinning hair than in males without thinning hair in both frontal and occipital regions, but the differences were not significant. Histologic and hormonal findings suggest that senescent thinning is a diffuse process that is histologically similar to AGA, but hormonally different and may not be entirely androgen dependent. We hypothesize that senescent alopecia is distinct from AGA.

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Guidelines and Techniques for the Accurate Classification of Chemotherapy-Induced Hair Follicle Dystrophy

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In order to recognise even subtle forms of hair follicle dystrophy after pharmacological manipulation, it is critically important to be able to identify and accurately classify the distinct stages of the follicular response to damage and the corresponding degree of hair follicle dystrophy. Here, we have developed a set of simple and pragmatic classification criteria for chemotherapy-induced hair follicle dystrophy, using the C57BL/6 J mouse model of cyclophosphamide-induced alopecia. We suggest to divide murine hair follicle dystrophy into six distinct stages: healthy anagen VI (HA), light dystrophic anagen VI (LDA), moderately dystrophic anagen VI (MDA), severe dystrophic anagen VI (SDA), dystrophic catagen (DC), and dystrophic telogen (DT). The basic classification criteria include: the structure and pigmentation of the hair shaft (continuous shaft and normal pigmentation during HA and LDA; broken shaft and disrupted pigmentation during SDA, DC and DT), the location and volume of ectopic melanin granules, the location, volume and shape of the dermal papilla (large and oval during HA and LDA; swollen during SDA; round and compacted during DC and DT) as well as abnormal widening of the hair canal (DC, DT). In addition, the following immunohistochemical markers aid in dystrophy classification: the number of TUNEL + keratinocytes in the bulb as a marker for the level of dystrophy-associated apoptosis (HA: no; LDA 3; MDA 5, DC 10), and the immunoreactivity for neural cell-adhesion molecule (NCAM) as well as alkaline phosphatase activity as a marker for the level of damage to the DP. These staging parameters not only offer a useful tool in murine models for chemotherapy-induced alopecia, but also when screening drug-treated mice for even discrete hair follicle dystrophy in a highly standardised, reproducible, easily applicable, and quantifiable manner.

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Complex Cytogenetic Rearrangement of Chromosome 8q in a Case of Ambras Syndrome

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Ambras syndrome (AMS) is a unique form of congenital universal hypertrichosis. In patients with this syndrome, the whole body is covered with fine, long vellus hair, except for areas where no hair normally grows. There is accompanying facial dysmorphism and abnormalities of the teeth, including retarded first and second dentition, and absence of some teeth. No endocrine or metabolic defect has been detected. Multiple affected family members suggest a genetic basis for the syndrome. Ambras syndrome has been found in association with an inversion of chromosome 8 in two isolated cases. One of these patients was reported in association with an apparently balanced paracentric inversion of chromosome 8 (8)(q12q22). Our cytogenetic analysis of this patient revealed that the rearrangement is more complex than initially reported. We found a relatively large deletion in 8q13, as well as a smaller deletion in 8q23. We also detected an insertion of the q23-q24 region into a more proximal region of the long arm of chromosome 8. Given the large number of breakpoints and the presence of substantial deletions, it is surprising that the proband did not exhibit phenotypic features other than those characteristic of Ambras syndrome.

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The Cytokeratin Profile of Loose Anagen Syndrome

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Loose anagen syndrome (LAS) typically presents in early childhood with significant hair loss. LAS is associated with easily removable anagen hair follicles and tends to improve with age. Histologic examination demonstrates a noninflammatory alopecia in which intracellular discohesion with cleft formation is identifiable between various follicular epithelial layers. Likewise, epidermolysis bullosa simplex (EBS) also presents as a noninflammatory disease, which tends to improve with age. The histology of EBS demonstrates similar intracellular discohesion with cleft formation involving epidermal epithelial layers. EBS is associated with a genetic abnormality leading to abnormal keratin 5 and keratin 14 formation. Henceforth, we decided to study the cytokeratin (CK) profile of LAS to see if an analogous process could explain the pathophysiology of this condition. Immunohistochemistry using antibodies to CK5/6, CK7, LMWK, CAM5.2, CK14, HWMK, CK20, and AE1/3 was applied to two cases of clinically proven LAS. The follicular epithelium of normal anagen follicle and loose anagen syndrome demonstrated a similar cytokeratin-staining pattern. Follicular epithelium reacted with CK5/6, CK14, HMWK, and AE1/3 and failed to stain with CAM5.2, CK20, CK7 and LMWK. In conclusion, the cellular discohesion of LAS does not appear to be related to abnormal follicular cytokeratins.

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Hair-Growth Promotion of Stigmastanol Maltoside: Experimental and Clinical Studies

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The sterols isolated and identified by fractionating the medicinal plants from India showed hair-growth promoting effects in mice. To enhance water solubility and skin penetration, the sterols were glycosylated. Among the various glycosylated sterols tested, Stigmastanol Maltoside (SM) was found to be most promising in promoting hair growth. SM significantly promoted hair growth in the cultured rat vibrissa follicles. The expression of acidic fibroblast growth factor (aFGF) was especially enhanced in the outer root sheath of the cultured rat vibrissa follicles, which was confirmed by immunohistochemical staining. When SM was applied to the dorsal skin of 9-week-old C3H mice, on which anagen stage transition induced by hair shaving was inhibited by the topical application of dihydrotestosterone (DHT), the inhibitory effect of DHT was cancelled and hair growth was promoted. The mRNA expression of aFGF, confirmed by *in situ* hybridization, was reduced by the application of DHT on the mice dorsal skin, which was also cancelled by the application of SM. From the results obtained, it is suggested that the sustained expression of aFGF in hair follicles by the antiandrogen-like action of SM promotes hair follicle formation, resulting in hair growth. Precise mechanism of the action of SM is being investigated. Clinical studies of the SM on alopecia androgenetica patients were conducted. Treatment of the patients with 0.1% SM tonic lotion for a four-month period was found to be as effective as the treatment using 1% Minoxidil. These findings clearly suggest that SM is an effective active ingredient for hair-growth preparations.

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Species-Specific Expression of Stearoyl-CoA Desaturase (SCD) in the Pilosebaceous Unit

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Stearoyl-CoA Desaturase (SCD) is the major enzyme that produces endogenous monounsaturated fatty acids. It plays important roles in lipid metabolism, membrane fluidity, and signal transduction. Previous studies from our laboratory indicate that three SCD genes are expressed in mouse skin. The lack of SCD1 expression results in defective growth of the pilosebaceous unit (PSU) in the asebia (ab) mouse. These findings implicate the importance of SCD in skin biology and hair growth. This comparative study was designed to determine the possible functions of SCD in human pilosebaceous unit. SCD was highly expressed in human hair follicle, especially in matrix keratinocytes, cortex, cuticle of the hair shaft and cuticle of internal root sheath (IRS). SCD was also detected in the inner layer of outer root sheath (ORS) and in eccrine sweat glands. Except for a few cells, the sebaceous gland was devoid of SCD mRNA expression. This pattern is dramatically different in mouse and pig where SCD is highly expressed in sebaceous and meibomian glands. Additionally, some apocrine glands express SCD in pig. This difference in SCD expression pattern may not be related to the number of SCD genes. Mouse has at least 3 functional SCD genes, whereas human has one functional SCD gene and pig may have only one SCD locus. These results suggest that SCD may have both conserved (sweat gland) and nonconserved (hair bulb) functions in the PSU and skin.

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Sebaceous Gland Apoptosis Induced Selectively by Liposomal 5-α-Reductase Inhibitor in Hamster Model

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Numerous side-effects occur from current acne therapy. Thus, there is a need for developing a selective, effective, topically applied 5-α-reductase inhibitor to modify pathological processes in the pilosebaceous unit. 5-α-reductase plays a critical role in normal and pathological androgen-dependent processes, including acne. The 5-α-reductase inhibitor, finasteride, is currently used as an oral treatment for specific inhibition of type II 5-α-reductase for androgenetic alopecia as well as for benign prostatic hypertrophy (BPH). However, the potential for systemic side-effects from oral finasteride is dose limiting and therefore decreases efficacy. We have previously developed a selective liposome hair follicle targeting system. We demonstrate in this report that the 5-α-reductase inhibitor N,N-diethyl-4-methyl-3-oxo-4-aza-5a-androstane-17β-carboxamide (4-MA) incorporated into liposomes induces apoptosis and inhibits growth of the dihydrotestosterone (DHT)-dependent hamster flank organ sebaceous gland. We have compared topical application of liposome 4-MA and solvent-formulated 4-MA and observed selective efficacy of topical application of liposome 4-MA by the reduction of size and induction of apoptosis only in the treated hamster flank organ. Apoptosis induced by liposome 4-MA in the treated flank organ sebaceous gland cells was observed both by fluorescent assays for DNA fragmentation (TUNEL) and by observation of condensed and fragmented nuclei. When 4-MA was topically applied formulated in ethanol and glycerol, the selective efficacy was lost. Topical liposome 4-MA did not significantly affect prostate weight, T/DHT ratios or body weight gain compared to controls indicating safety as well as efficacy. Liposome 4-MA will be further developed as a topical agent to treat acne as well as androgenic alopecia.

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Decreased Serum Ferritin is Associated with Alopecia in Women: A Case-Control Study

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Hair loss in women is a common problem. Observational data have suggested that alopecia in women may be associated with decreased body iron stores. Our aim was to evaluate whether common types of alopecia (telogen effluvium, alopecia areata, and androgenetic alopecia) are associated with decreased tissue iron stores (as measured by serum ferritin) utilizing a case-control methodology. Cases were obtained by selecting 45 consecutive patients treated at the University of Pennsylvania hair and scalp clinic in the year 2000. Patients with elevated erythrocyte sedimentation rates were excluded. Control patients consisted of individuals without hemochromatosis gene (HFE-1) mutations who presented to the Hospital of the University of Pennsylvania Division of Medical Genetics. Data were analyzed utilizing the unpaired Student's t test assuming unequal variances in order to assess whether the mean ages of controls differed from the mean age of each individual case group. The t test was then used to determine whether the ferritin level in each of the individual alopecia groups was lower than the mean ferritin level in controls. The ages of cases and controls did not differ significantly when grouping all cases together or when comparing individual case groups to the controls, suggesting that any differences in ferritin levels between cases and controls are not secondary to differences in age. Using the t test, we found that the mean ferritin level (stated as ng per ml [95% confidence intervals]) in cases with androgenetic alopecia (31 [14, 48]), telogen effluvium (39 [24, 55]) and alopecia areata (21 [5,38]) were statistically significantly lower than levels in normal controls (61 [38, 84]), with p-values of 0.015, 0.049, and 0.003, respectively. This is the first study to utilize a case-control methodology to address the relationship between ferritin levels and alopecia, and our findings may have implications in terms of therapeutics and developing a better understanding of the etiology of these

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Binding of Oncogenic Human Papillomavirus (HPV) 16 Capsids to Keratinocytes is Independent of α-6 Integrin Expression

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Infection with mucosotropic human papillomaviruses (HPV) causes benign and malignant epithelial lesions of the anogenital mucosa. The cellular receptor for HPV has not yet been identified due to difficulties to obtain infectious virions and the lack of a permissive tissue culture system, and thus the determinants for the tissue and species specificity of papillomavirus infection remains elusive. Recently, α-6 integrin has been proposed as a binding protein for the low-risk HPV6 using the transformed human keratinocyte cell line HaCaT. To determine if high-risk HPV infection is dependent on α-6 integrin expression, we have used HPV16 virus-like particles (VLP) expressed in insect cells to determine binding of a high-risk HPV capsid to (SV-40 transformed) "normal" or α-6 integrin deficient human keratinocytes or HaCaT cells. The absence of α-6 integrin expression was determined by FACS using GoH3 antibody. Using biochemical assays binding of HPV16 VLP to cell lines was detected independent of cell-surface expression of the α-6 integrin. Binding of VLP was inhibited by preincubation with heparin or antibodies directed against neutralizing and conformation-dependent surface epitopes of HPV16, but not by control antibodies directed against the heterologous HPV6. In addition, preincubation of "normal" keratinocytes with antibodies to α-6 or β-4 integrin did not reduce HPV16 VLP to cell binding. Together these data indicate that the α-6 integrin is not required for HPV16 binding to the natural host cell and thus is not a primary candidate for the evolutionary conserved cell surface receptor for oncogenic HPV types.

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Non-Invasive Topical Immunization by a Peptide Vaccine Against Hepatitis B and Rubella Virus

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Immunogenic peptides are excellent tools in vaccine research since they are extensively used for definition of T- and/or B-cell epitopes. Topical delivery of peptides for immunization is attractive because it offers convenience, increased patient compliance, and reduced costs. Progress in topical peptide vaccination, however, has been hindered by concerns about penetration through the epidermal permeability barrier. We applied immunogenic peptides to nonpretreated normal back skin of immune competent mice. Ruby 9, a nine amino acid peptide from rubella virus protein E2, and HBsAg 24mer, a sequence between residues 124 and 147 of hepatitis B surface antigen (HBsAg), induced specific serum IgG antibodies without cross-reactivity to each other or to other irrelevant peptide controls. Induction of specific immune responses was observed whether or not immunogenic peptide was coupled to a oligoarginine protein transduction sequence (PTS). In each case 5 mice were treated with the peptide in aqueous solution in the presence and absence of topical adjuvant (cholera toxin). In both cases cholera toxin did not seem to enhance the immune responses. Interestingly, in the case of HBsAg 24mer, specific serum IgG were approximately 3 fold higher than those produced by intramuscular injection of the commercially marketed recombinant HBsAg vaccine. Antibody levels in the case of rubella were in the same order of magnitude as those produced by intraperitoneal injection of the commercial live rubella virus vaccine. These topical immune responses could be achieved with minute amounts of antigen. As little as 50 ng of topically applied peptide could elicit specific antibody production. These data demonstrate induction of immune responses in animals via simple, painless and economical application of immunogenic peptides to intact skin.

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Induction of HIV-1 Replication by Human Herpesvirus-8 (HHV-8)

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HIV-1 infected individuals are commonly infected with herpesviruses including cytomegalovirus, herpes simplex virus, varicella-zoster virus and HHV-8. Previous studies have shown that coinfection with herpesviruses can modulate HIV-1 replication. This can occur either through a direct interaction between the two viruses infecting the same cell or through a secondary stimulation by cellular factors released in response to the herpesvirus infection. In addition, a reciprocal interaction can occur resulting in altered herpesvirus replication in the presence of HIV-1. HHV-8 is the newest member of the γ2-herpesvirus family and has been consistently associated with Kaposi's sarcoma. As AIDS-related KS is well recognized as a particularly aggressive form of the disease, we have begun a series of studies to investigate the potential interaction between HIV-1 and HHV-8. We have recently demonstrated that coculture of HIV-1 infected T cells with cell lines latently infected with HHV-8 results in HHV-8 replication, a response that was mediated, at least in part, by inflammatory cytokines. In the present study, we investigated HIV-1 replication in the presence of HHV-8 in both an *in vitro* and *in vivo* model system. The results demonstrate a significant increase in HIV-1 replication following culture of HIV-1 infected T cells in the presence of HHV-8 infected B cell lines, but not control, uninfected B-cells. In addition, HIV-1 replication in T-cells injected into human skin transplanted on SCID mice was significantly increased in mice also injected with purified HHV-8, but not in animals injected with PBS as a control. While the mechanism responsible for induction of HIV-1 replication remains to be identified, the results from our *in vivo* model system involving normal human skin transplanted on immunodeficient mice indicate that immune responses to the injected virus would be minimal and that the effects are more likely due to endogenous factors and not the result of recruitment of inflammatory cells into the skin. These results also indicate that these two viruses may interact at the molecular level in coinfected patients, which may underscore the unexpectly high frequency and aggressive nature of AIDS-related KS.

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Chemically Modified RANTES Analogues Block CCR5-Mediated HIV Infection of Langerhans Cells Within Epithelial Tissue Explants: Implications for Blocking Sexual Transmission of HIV

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Initial biologic events that underlie sexual transmission of HIV are poorly understood, although it is believed that mucosal Langerhans cells (LC) are initial targets for virus. Furthermore, it is known that the majority of transmitted HIV strains utilize CCR5 as a coreceptor for entry into cells (labeled R5 viruses). To model these events, we recently reported that LC within epithelial tissue explants could be preferentially infected with R5 HIV isolates (Kawamura *et al*, *J Exp Med* 2000). Here, to help elucidate the most potent inhibitor of HIV infection in LC, we directly compared the HIV blocking ability of four chemically modified chemokines that bind CCR5 (AOP-RANTES, NNY-RANTES, QST-DIH-RANTES, UCB-RANTES) using our explant model. As quantified by intracellular HIV p24 staining and flow cytometry of LC, all analogues could block R5 HIV in a dose-dependent manner (1–100 nM) when preincubated with explant tissue for 20 min prior to HIV exposure. In addition, all analogues could inhibit LC-mediated HIV infection of CD4+ T cells that were cocultured with LC. Importantly, the newer analogues (NNY-RANTES, QST-DIH-RANTES, and UCB-RANTES) performed at least 10-fold better than AOP-RANTES. In summary, we document R5 HIV infection in single LC following exposure to virus within epithelial tissue and show that this infection can be blocked by preincubation of tissue with drugs that compete with virus binding and entry into cells. Importantly, these results suggest that a topical microbicide containing a chemically engineered RANTES analogue may be a useful agent to block sexual transmission of HIV.

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HIV Envelope Glycoprotein gp120 Secreted by HIV-Infected Dendritic Cells Impairs T Cell Proliferation and IL-2 Production: Implications for HAART-Treated Patients

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It is known that highly active antiretroviral treatment (HAART) for HIV-infected patients often leads to complete suppression of plasma viremia, yet it is not known why HAART commonly fails to completely restore immune function. Our laboratory has long been interested in the role of dendritic cells (DC) in the immunopathogenesis of HIV disease, and here we examined the mechanisms underlying HIV-mediated dysfunction of DC. HIV-infected DC were significantly impaired in their ability to stimulate proliferation (mean stimulation indices = 0.46 and 0.24 for macrophage-tropic HIV_{BaL} and T cell line-tropic HIV_{IIIB}, respectively) and IL-2 production (mean production vs. controls = 0.66 and 0.45 for the same 2 viruses) in cocultured allogeneic CD4+ T cells. MHC class II and costimulatory molecule expression was normal on HIV-infected DC and, consistent with this, paraformaldehyde-fixed HIV-infected DC exhibited similar antigen presenting cell (APC) capacity when compared to fixed uninfected DC. Interestingly, HIV-infected DC still exhibited poor APC capacity in the presence of potent combinations of antiretroviral drugs (the reverse transcriptase inhibitors AZT and ddI and the protease inhibitor JE-2147) that blocked transfer of HIV infection to T cells. However, when soluble CD4 (capable of binding to gp120) was added to DC-T cell cocultures, APC capacity of HIV-infected DC was completely restored. Western blot analysis and ELISA of supernatant proteins revealed that antiretroviral drugs blocked mature HIV virion production (81–100% inhibition), but not gp120 production (contained in immature defective virions). In summary, HIV-infected DC induced T cell dysfunction through a gp120-mediated negative growth signal, a process that was blocked by soluble CD4, but not by conventional antiretroviral drugs. These findings may be important in understanding immune dysfunction in the setting of HAART and may contribute to the design of novel immunotherapeutic approaches for HIV disease.

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Direct Involvement of Human Immunodeficiency Virus Type I in Kaposi's Sarcoma-Associated Herpesvirus (KSHV) Reactivation

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Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus etiologically linked to primary effusion lymphoma (PEL), to a subset of multicentric Castleman's disease, and to Kaposi's sarcoma (KS), the most common neoplasm associated with AIDS. Among KSHV-infected individuals, the risk of KS is much higher in HIV-1-infected ones than among those with other types of immunosuppression, which could suggest a direct action of HIV-1 on KSHV replication. We show that BC-3 cells, a chronically KSHV-infected B cell line, are permissive to HIV-1. In these cells, HIV-1 infection leads to reactivation of latent KSHV genomes, as demonstrated by the expression of KSHV lytic viral mRNAs. While recombinant HIV-1 gp120 fails to enhance herpesvirus expression, transient transfection of the HIV-1 *trans*-activator Tat suffices to reactivate latent KSHV. By showing that HIV-1 infection directly reactivates latent KSHV, our results suggest a direct role of HIV-1 on the onset of KS in coinfected individuals.

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A Wound-Isolated Pseudomonas Aeruginosa Grows a Biofilm *In Vitro* and is Visualized by Light Microscopy

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In chronic wounds, biofilms probably play a vital role in protecting bacteria from host defenses and antimicrobial medications by creating a barrier of exopolysaccharide that is difficult for the immune system and antibiotics to penetrate. A biofilm is an exopolysaccharide matrix that is produced and secreted by certain species of bacteria. The exopolysaccharide from many nearby bacteria can convalesce and form a thick slime layer, or biofilm, that shelters the bacteria from the immune system and antibiotics. Biofilms have been found to exist in many diseases; most notably, cystic fibrosis that is chronically infected with a biofilm-secreting Pseudomonas aeruginosa. The goal of this study was to visualize by light microscopy a growing biofilm secreted by Pseudomonas aeruginosa that was isolated from a burn wound. Pseudomonas aeruginosa was added to 200 ml of sterile Tryptic soy broth to create a concentration of approximately 2.2×10^5 organisms per milliliter of nutrient broth. This concentration correlates with 10^5 colony-forming units that some clinicians use to define the presence of an infection. Clean plastic cover slips were washed in isopropyl alcohol and then suspended in the Pseudomonas-innoculated Tryptic soy broth and allowed to grow in a 37°C shaker. Cover slips were carefully removed with sterile instruments at 3, 5, 7, 8, 9, 10, 11, 12 and 24 h, washed on one side with isopropyl alcohol and a sterile cotton swab, and placed on glass slides. Slides were stained with a modified Congo Red/Ziehl Carbol Fuschin staining technique. The biofilm stained orange/pink and the cells stained purple/red. The slides demonstrate biofilm growth that initially shows exopolysaccharide surrounding only individual Pseudomonas bacteria, and then the exopolysaccharide grows and merges with that of many nearby bacteria to form a thicker slime layer, which is the biofilm. This staining method may be used to detect biofilms in samples obtained from chronically infected wounds.

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Gram Negative Staphylococci Isolated from the Warm Facial Skin of Patients with Rosacea Secrete Excessive Lipase at Higher Temperatures

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Pustules on the faces of patients with rosacea suggest a bacterial infective process, and indeed they often resolve after treatment with topical or systemic antibiotics. However, no abnormal bacteria have been isolated from the skin surface or from the pustules. Bacteria of the normal skin flora might produce pustules if they behaved abnormally in the warmer-than-normal milieu of red and flushed facial rosacea skin. We hypothesized that these bacteria might produce different virulence proteins at these different temperatures. Coagulase-negative bacteria were isolated from pustules of patients with untreated rosacea. Bacteria were cultured at both 30°C and 37°C. Growth rates were identical. All isolates secreted more protein at 37°C. One protein was highly up-regulated at 37°C. This protein was the dominant secreted protein from all four isolates on all gels. The protein was isolated and sequenced and identified as a lipase. Its lipase gene was up-regulated by elevated temperatures and by the presence of oxygen in the late logarithmic phase; the lipase was predominantly made when organisms were begining to encounter nutrient depletion. As controls, four coagulase negative staphylococci were isolated from the skin surface of individuals without rosacea. Two of the four isolates also secreted the lipase and two did not. Bacteria behave differently at different temperatures. Normal constituent bacteria of the skin surface and pustules may become pathogenic when they secrete abnormal or abnormal amounts of temperature-dependant virulence factors. Abnormal activities of bacteria could create pustules of rosacea and the "dryness" often associated with rosacea (rosacea dermatitis).

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Lifestyles of Bacteria in Wounds: Presence of Bioflims?

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Biofilms consist of bacteria and other organisms that live within a matrix of extracellular polysaccaride (EPS) and have been implicated in bacterial diseases such as otitis media, dental plaque and cystic fibrosis. The purpose of this study was to examine wounds for the formation of bacterial biofilms. Partial thickness wounds were made on three pigs with a dermatone. Wounds were challenged with Pseudomonas aeruginosa and covered with either a polyurethane dressing or plastic cover slip. At 72 h each wound was vigorously flushed three times with sterile saline to dislodge any nonadherent bacteria. The flushed wounds were then cultured with a surfactant solution using a scrub technique. Both the flushed and scrubbed samples were plated on Pseudomonas isolation agar for quantitation. Cover slips were removed from the wounds at 72 h and wound curettage was obtained. Congo red staining procedure, which detects EPS was used to stain both. A thick dark red to a yellow orange amorphous EPS matrix was seen surrounding bacteria indicating a biofilm. Wounds cultured with saline or surfactant demonstrated that there were two distinct populations of bacteria living in the wound area. The nonadherent population displayed a variation from wound to wound whereas the adherent population had a narrower range suggesting some sort of critical mass for those bacteria, which adhered to the wound. This preliminary work has demonstrated that bacterial biofilms do form in wounds. This *in vivo* assay system will provide a means to examine therapeutic modalities for bacteria living in a protective biofilm.

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Are Biofilms Present in Chronic Wounds?

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Biofilms are made up of groups of microorganisms, which live within a polysaccharide matrix and are adhered to biological or nonbiological surfaces. Organisms that live within biofilms are resistant to both host defense mechanisms and to systemic and topical antimicrobial agents and have been implicated in many bacterial diseases, such as otitis media, cystic fibrosis, and tuberculosis. Chronic wounds have been shown to harbor many species of pathogens in colonized and clinically infected wounds. The association between chronic wounds, the organisms they contain and the mechanisms to prevent infection and promote wound healing are major concerns. We hypothesize that chronic wounds that are frequently covered by fibrinous tissue could provide an excellent matrix for the formation of microbial biofilms. Two patients with nonhealing wounds, with signs of infection, were cultured. Cultures on both patients grew pseudomonas. Both patients were treated with antibiotics according to sensitivity as per antibiogram, but neither wound improved. A preliminary evaluation of these two chronic human wounds for biofilm formation is reported here. After receiving patient's informed consent, the two chronic wounds were scrapped with a blade to remove the fibrinous tissue. The curetted tissue was smeared onto a glass slide and preserved in 10 mM cetylpyridinium chloride. The tissue was stained with a modified Congo red staining technique. Bacterial cells were observed in the smear and stained dark red. An orange pink material was also observed surrounding the bacteria, presumptively indicating the presence of an extracellular polysaccharide material, biofilm. Further studies are planned to confirm these finding and to develop treatment strategies for bacteria living within biofilms in chronic wounds.

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Increased Numbers of Methicillin Resistant Staphylococcus Aureus Isolated from Pediatric Impetigo Lesions in a Public Health Clinic

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Over the last decade we have observed significant increasing levels of antibiotic resistance in Staphylococcus aureus isolates from impetigo lesions in otherwise healthy patients in San Juan, Community Health Center In 1989 S aureus was isolated from 46 of the 53 patients cultured and susceptibility to 10 antibiotics was determined by a modified Bauer Kirby disc diffusion. Five percent of the isolates were resistant to methicillin. In a similar population and site in 1993, six percent of the isolates from 30patients were resistant to methicillin. We report here the results of cultures from a large cohort of impetigo patients from the same local Community Health Center in the year 2000. To this date 193 patients have been cultured and 170 patients were culture positive for either S. aureus and or Streptococcus pyogenes. Ninety percentage of culture positive lesions grew S. aureus, which continued to be the most prevalent pathogen in this community setting. Direct plating techniques were used to recover Streptococcus with selective media of blood and crystal violet. Twenty-two percentage of positive lesions contained both staphylococcus and streptococcus and 10% were positive for streptococcus only. There was an increase in MRSA to 12% of the total 153 Saureus culture positive patients. When Methicillin resistance was evaluated for patients that grew only S. aureus, the rate of resistance climbed to 15%. This alarming increase (300% in 11 years) of MRSA from community pediatric impetigo patients may have surpassed the trends seen in other community acquired MRSA infections.

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Hyphae and Yeast of *Candida Albicans* Differentially Regulate IL-12 Production by Human Blood Monocytes: Inhibitory Role of *Candida Albicans* Germination

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Interleukin-12 (IL-12) is essential for inducing type 1 immune responses, and the development of IFN-γ producing T cells, which in turn are associated with host resistance to candidal infection. Early work from our group demonstrated that *Candida albicans (CA)* and less virulent *C. krusei* differentially induced IL-12 production by human monocytes, with *CA* inability of inducing the production of this cytokine. In this study, we further explore the mechanism/s involed in this inhibition process focusing on the role of yeast-to-hyphal transition. To investigate the influence of germination on production of IL-12 by monocytes, we incubated monocytes with isogenic strain pairs of *C. albicans* that differ in their ability to germinate and the yeast *Saccharomyces cerevisae.* Following incubation, supernatants were collected and the levels of IL-12 determined by ELISA. Additionally, the effects of incubating monocytes with different strains on IL-12 mRNA expression levels was also assessed. Our data showed that unlike *S. cerevisae*, which induced IL-12 production by monocytes (162 ± 41 pg per ml, $n = 3$), live germinating *C. albicans* failed to induce this cytokine (23 ± 7 pg per ml, $n = 7$). Moreover, *C. albicans* suppressed IL-12 production induced by heat-killed *C. albicans*. Monocyte viability assay revealed that inhibition of IL-12 production is not due to monocyte killing by *C. albicans*. Comparison of the ability of germinating *C. albicans* parent strain, HLC54 germinating deleted mutant, and the germinating HLC84 revertant strain, to inhibit/induce IL-12 production by monocytes showed that both the parent and the revertant strains failed or partially to induce IL-12 production (23 ± 7 pg per ml and 223 ± 16 pg per ml, respectively). In contrast, the nonfilamenting deleted mutant induced significantly higher IL-12 production than the other two filamenting strains ($p < 0.05$). Our data indicate that *C. albicans* modulates IL-12 production by monocytes through multiple mechanism/s, in which germination plays a critical role in inhibition of IL-12 production.

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***In Vivo* Confocal Microscopy, a Noninvasive Tool for Quantitative Evaluation of Comedolysis**

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Near-infrared reflectance confocal microscopy (CM) provides noninvasive real-time images of *en face* tissues sections with high resolution and contrast to a depth of 200–350 μm. This study was designed to evaluate this evolving technique as a noninvasive imaging tool for monitoring comedolytic agents. We have utilized the rhinomouse model from Charles River (Portage, MI) and Jackson Laboratories (Bar Harbor, ME) in combination with topical application of comedolytic agents, *all-trans* retinoic acid (RA, 0.025% and 0.0025%) and *all-trans* retinol (Rol, 0.025%). *In vivo* CM was performed at baseline, one and two weeks during the treatment protocol. Major morphometric parameters for *in vivo* monitoring of comedolysis included diameter and numericle density of utricles. Our results showed significant morphometric differences among animals from Charles River Labs. After one-week application, the number and diameter of utricles significantly decreased in animals treated with 0.025%RA or 0.025%ROL. After two-week application, utricles disappeared in both groups. At that time, animals treated with 0.0025%RA showed a significant reduction in number but not in size of the utricles. In conclusion, *in vivo* CM represents a useful tool in the quantitative evaluation of utricles and could also be used for real-time noninvasive assessment of comedolysis induced by pharmacologic agents.

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Induction of Th1 Responses and Resistance to Leishmania major in Susceptible BALB/c Mice with Interleukin 4

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Resistance and susceptibility to intracellular pathogens are regulated by interleukin (IL-)12, which mediates Th1 responses and resistance, and IL-4, which induces Th2 cells and susceptibility. Thus, inhibition of IL-4 with mAb or injection of IL-12 can prevent the development of Th2 responses after infection with the intracellular parasite *Leishmania major (L. major)* and induce resistance in otherwise susceptible BALB/c mice. Here we show that, paradoxically, IL-4 can instruct dendritic cells (DC) to produce high levels of IL-12p70 and promote T cell differentiation towards a Th1 phenotype *in vitro* and *in vivo*. Treatment of susceptible BALB/c mice with IL-4 during the first 8 h of infection with *L. major,* prior to detectable ligand recognition by T cells, induces IL-12 dependent Th1 responses and resistance to L. major. In contrast, extending the action of IL-4 into the period of T cell activation results in Th2-differentiation and development of progressive leishmaniasis, even in resistant mice. Since immune responses evolve via the consecutive activation of first DC and, subsequently, T cells, the contrasting effects of IL-4 on DC and T cell differentiation can lead to the maturation of T cells with opposing functional phenotypes. These findings may impact on the design of cytokine-based immune therapies currently studied in humans with melanoma or psoriasis.

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In Experimental Leishmaniasis Deficiency of CD18 Results in Unimpaired IL12 Release by Macrophages, but also in a Dissociated Th1 Response

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We have previously shown that: (i) CD18–/–, but not WT mice, are susceptible in experimental leishmaniasis, and that (ii) neither decreased phagocytosis and leishmanicidal activity of CD18–/– macrophages nor reduced recruitment of T cells were sufficient to explain susceptibility. Thus, we now analysed the nature of the specific T cell response. In lymphnodes of infected CD18–/– mice we found very low production of IFN-γ and none of IL2 or IL4 (ELISA). After coincubation with bone marrow-derived DC pulsed with L.major-Ag, T cells from infected CD18–/– mice released intermediate amounts of IFN-γ, little IL4 or IL2, and they did not proliferate (3H-Thymidine incorporation). By adding IL-2 (10 ng per ml) we were able to bypass this proliferation deficit and to markedly increase release of IFN-γ exclusively by T cells from infected mice. Thus, infected CD18–/– mice mount a L.major-specific Th1-like response in which IFN-γ release and proliferation are dissociated. Since this Th1-like cytokine response developped surprisingly despite lack of CD11a/CD18 we searched for a costimulant which would substitute for LFA-1. Expression of B7-2, B7-1 or MHC II did not differ between infected CD18–/– and WT cells (FACS). However, release of IL12, which is usually suppressed after uptake of L.major by macrophages from susceptible as well as resistant mice, was not suppressed in CD18–/– macrophages (ELISA). Blocking different receptors for L.major revealed that parasites were only able to inhibit IL12 release when taken up via their major receptor CD11b/CD18. Neutralization of IL12 *in vivo* abolished emergence of IFN-γ releasing Th1-like cells. Thus (i) uptake of L.major by receptors other than CR3 prevents impaired IL12 release and helps to induce Th1-like cells despite lack of LFA-1 in CD18–/–mice; (ii) increased release of IL12, however, does not restore the reduced proliferation of CD18–/– Th1-like cells, so that their low number results in insufficient control of L.major infection in CD18–/– mice.

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A Role for the Antimicrobial Protein Granulysin in Host Defense in Leprosy

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A novel mechanism by wich T cells contribute to host defense against microbial pathogens is by release of the antimicrobial protein granulysin. We investigated the role of granulysin in human infectious disease using leprosy as a model. Granulysin-expressing T-cells were detected in cutaneous leprosy lesions, at a six-folder greater frequency in patients with the localized tuberculoid as compared with the disseminated lepromatous form of the disease. In contrast, perforin, a cytolytic molecule that colocalizes with granulysin in cytotoxic granules was expressed at similar levels across the spectrum of disease. Immunofluorescence labeling of leprosy lesions, revelead that cells containing granulysin were CD3+ T cells and expressed the CD4 but not the CD8 marker.We found that CD4+ T-cell lines derived from tuberculoid skin leprosy lesions lysed antigen-pulsed targets by the granule exocytosis pathway, expressed perforin and granulysin and reduced the viability of mycobacteria in infected targets. These data point for a novel role for CD4+ T cells in host defense and provide evidence that T cell release of granulysin contributes to host defense at the site of infection in human disease.

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Cathelicidin Antimicrobial Peptides are a Critical Defense Mechanism Against Group A Streptococcal (GAS) Necrotizing Cellulitis

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Recent discoveries in mammals of peptides with *in vitro* antimicrobial activity have led to theories that these molecules are important to innate immune defense. Cathelicidins are one family of such peptides that are directly expressed in keratinocytes and other epithelial and lymphoid cell types. Humans and mice contain a single cathelicidin gene whose mature products are the structurally similar peptides LL-37 and CRAMP, respectively. To test if cathelicidins are important for cutaneous defense against the common human pathogen GAS, we generated mice null for the gene encoding CRAMP. Within 6 days of sc injection of wild-type GAS, wild-type 129/SVJ mice (+/+) develop lesions of necrotizing cellulitis measuring from 4 to 9 mm^2, –/+ littermates develop larger lesions (8–12 mm^2), and –/– animals have the most severe infections (14–21 mm^2). Tissue biopsy and quantitative culture of lesions 7 days after injection show +/+ mice have few colonies of GAS while –/+ and –/– have active infection, with ca 2X more colonies per mg tissue in –/– than –/+ mice. To confirm the apparent conclusion that CRAMP limits GAS growth in skin, we used random transposon mutagenesis to produce 2 genetically distinct GAS clones resistant to inhibition *in vitro* by either LL-37 or CRAMP. Injection of the resistant GAS clones in normal Balb/c (+/+) mice resulted in significantly larger lesions and greater bacterial survival at 5 days than did injection of wild type GAS in identical animals. These observations are the first proof for mammals that antimicrobial peptides play a role *in vivo* to prevent infection. Our findings suggest that the expression and function of cathelicidins limits bacterial pathogenicity and works together with other components of immune defense to clear infection and prevent disease.

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The Expression of Functional CD14 and Toll-Like Receptors on Human Keratinocytes

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CD14 and Toll-like receptors (TLRs) have been known to play an important role in LPS-mediated cellular responses in bacterial infections. Although CD14 and TLRs expression has been demonstrated on a number of myeloid cells, little is known about the expression and function of these LPS receptors on nonleukocytes such as keratinocytes. In this sutdy we tested the possibility that the human keratinocytes are capable of expressing both functional CD14 and TLRs. Using quantitative RT-PCR and Northern blot, we found that the cultured human keratinocytes constitutionally express CD14 and TLR2/TLR4 mRNA, and that LPS treatment (100 ng per ml) increases the mRNA expression of these receptors. Cell surface expression of CD14 and TLR4 was verified by FACS analysis. To assess the functional activity of these receptors in human keratinocytes, we measured intracellular Ca^{++} and NF-κB activity in response to LPS. LPS treatment triggered a rapid induction of human keratinocyte intracellular Ca^{++}, which could be blocked by specific antibodies to each receptor. The rapid increase in intracellular Ca^{++} in response to LPS was followed by the nuclear translocation of NF-κB. This study indicates for the first time that cultured human keratinocytes express both functional CD14 and TLRs which can be up-regulated by LPS. These results have important implications for our understanding of the pathophysiology of cutaneous infections.

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Activation of Human Dendritic Cells by Platelet Activating Factor is Maturation Dependent

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Dendritic cells (DCs) are characterized by their ability to migrate into target sites, process antigens and activate naive T cells. Effector functions induced by platelet activating factor (PAF) and the cytokine macrophage inflammatory protein 3-β (MIP3-β) as well as the mRNA-expression of their receptors were analyzed in human DCs during lipopolysaccharide (LPS)-promoted maturation. Immature human dendritic cells were differentiated *in vitro* from monocytes with interleukin-4 and granulocyte macrophage colony stimulating factor. Platelet activating factor induced calcium transients, actin polymerization and chemotaxis in these cells. In addition, RT-PCR experiments indicated mRNA-expression of the PAF receptor. Cell studies and mRNA analyses further revealed that immature DCs neither respond to MIP3-β nor express its specific receptor, called CCR-7. Induction of cell differentiation by LPS lead to the loss of the mRNA-expression of the PAF receptor accompanied by decreasing intracellular calcium release, actin polymerization and migration after stimulation with PAF. In contrast, LPS treatment induced increasing responsiveness towards MIP-3β and mRNA expression of CCR-7. Comparable data regarding mRNA expression of PAF receptor and PAF-responsiveness were also obtained with an other maturation protocol using TNFα instead of LPS. The direct comparison between the two different protocols showed a slower decrease of PAF-responsiveness induced by TNFα compared to LPS. These results show the loss of PAF responsiveness together with down-regulation of PAF receptor mRNA expression during maturation of human DCs. Therefore, these findings point to a functional relevance of PAF in recruiting immature DCs, whereas MIP3-β might regulate the migration of DCs to secondary lymphoid organs.

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Induction of Proinflammatory Cytokines by Activation of the Capsaicin Sensitive Vanilloid Receptor VR-1 on Human Keratinocytes

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Capsaicin, one of the pungent ingredients of hot chili peppers, induces a subpopulation of sensory neurons to release neuropeptides by activating the recently cloned vanilloid receptor-1 (VR-1). Vanilloid receptors are also triggered by acidic and thermal stimuli that could occur during cutaneous inflammation and wound healing. To date, in the skin, VR-1 receptors have only been identified on cutaneous nerves. The aim of this study was to examine the direct effect of capsaicin on keratinocyte proinflammatory cytokine production and to determine if this effect is mediated by the VR-1 vanilloid receptor. Our results demonstrate for the first time that normal human keratinocytes express functional VR-1 receptors. Real time quantitative RT-PCR studies indicate that VR-1 mRNA is constitutively expressed in human keratinocytes and is not modulated by capsaicin treatment. Furthermore, capsaicin (0.1–100 mM) induced up to a 25-fold expression of the proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α. Pretreatment of keratinocyte cultures with capsazepine, a specific competitive antagonist of vanilloid receptors, prior to capsaicin, completely blocked cytokine mRNA expression to control levels indicating that capsaicin induces the expression of keratinocyte proinflammatory cytokines by activating the VR-1 vanilloid receptor. Thus, our data indicate that the activation of capsaicin-sensitive vanilloid receptors located on keratinocytes may directly promote cutaneous inflammatory responses. These results have important implications for the role of vanilloid receptors in normal and diseased skin.

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Local Skin Lesions in Rat After Asubcutaneous Deposition of Capsaicin

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This study describes the time course of development, regional distribution and innervation of spontaneous lesions in adult Sprague-Dawley rats desensized to capsaicin. In one group, capsaicin was administered subcutaneously by injection in the neck skin. In the second group capsaicin was injected with a long needle in the skin of the back skin and deposited in the subcutaneous fat of the neck. Occurrence and distribution of dermal nerve fibers was monitored using immuno-histochemistry with antibodies against calcitonin gene-related peptide (CGRP) and a pan-neuronal marker, protein gene product 9.5 (PGP 9.5). In the first group rats developed spontaneous wounds in the neck area. In the second group, lesions appeared in the skin of the back and occasionally in the neck area. Development of wounds in the afflicted areas was paralleled by a reduction of the CGRP immunoreactive nerve fibers by 80% in the first group and 72% in the second group. The number of PGP-immunoreactive fibers was reduced by 39% and 41% resp. The density of CGRP-IR innervation of the wound area was the same as in the adjacent, nonlesional skin in both groups. The healing of the spontaneous wounds was delayed compared to surgical wounds on control animals The wounds healed with hypertrophic scars which was paralleled by proliferation of CGRP in the skin of the back. The study shows that cutaneous lesions in rats systemically treated with capsaicin appear in the region of subcutaneous deposition of capsaicin. An even depletion of capsaicin-sensitive nerve fibers in the area of deposition suggests that an additional factor is needed to induce lesions. Hypothetically, an impaired nociception in the afflicted area may result in a more vigorous grooming behavior inducing a local skin damage.

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Cutaneous Inflammation Induced by Endothelin-1 is Mast Cell-Dependent

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We have recently shown, that endothelin-1 (ET-1), a potent vasomodulatory peptide, induces pronounced inflammatory responses in murine skin. Since mast cells (MC) express functional receptors for ET-1, we have used genetically MC-deficient KitW/KitW-v-mice to characterize the role of MC in ET-1 induced cutaneous inflammation. ET-1 (10^{-6} M, 20 µL, left ears) and vehicle (20 µL, right ears) were injected intracutaneously into the ears of KitW/KitW-v-mice and normal +/+ mice. Ear thickness was measured for 6 h at various time points after injection. Ear swelling in ET-1-injected ears was markedly reduced in the absence of mast cells (KitW/KitW-v - mice: 58 ± 6 µm vs. +/+ mice: 118 ± 5 µm at 1 h, $p < 0.001$). In addition, MC degranulation in ET-1-treated ears of +/+ mice was significantly increased 1 h and 6 h after injection as compared to vehicle treated control ears ($p < 0.05$). To test, whether the adoptive transfer of MC to the ears of MC-deficient KitW/KitW-v-mice would restore impaired inflammatory responses to ET-1, we injected ET-1 into KitW/KitW-v-mice reconstituted with bone marrow-derived cultured MC (left ears, KitW/KitW-v + BMCMCs) or vehicle (right ears, KitW/KitW-v + Veh). Earswelling in MC-reconstituted ears after ET-1 injection was significantly increased as compared to ET-1-treated control ears (KitW/KitW-v + BMCMCs: 100 ± 6 µm vs. KitW/KitW-v + Veh: 45 ± 5 µm at 1 h, $p < 0.001$) and virtually the same as in ET-1-injected ears of +/+ mice. These data suggest that ET-1 induced inflammation in murine skin is MC-dependent and thus provide a novel mechanism of ET-1 induced inflammatory responses.

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Characterization of Substances from the Itch Inducing Plant Macuna Pruriens

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An itch inducing ''proteinase'' termed mucunain was described as the active ingredient in the spicules of the seedpods of the cowhage plant Macuna pruriens. This work, by Shelley and Arthur, in the 1950s, was not continued. We have obtained spicules from Macuna pruriens and made extracts under both aqueous and organic conditions. The extracts have been analyzed using a variety of biochemical approaches including anion exchange, gel electrophoresis, thin-layer chromatography, reverse-phase HPLC and MALDI-TOF MS (matrix-assisted laser desorption time-of-flight mass spectrometry). The extracts and purified material have also been subjected to protease digestion without lost of the itching activity. Pruritic activity appears to be maintained in the whole extract and in at least one of the several peaks under reverse-phase HPLC. No peaks absorb at 280 nm. MALDI analysis has determined that the molecular masses associated with the peaks are between 200 and 1000 amu. No substance with the mass of histamine, 111, was noted. No bands are present on gel electrophoresis of the total extract or of the HPLC peaks. These results suggest that the active ingredient is not histamine and, in contrast to the results of Shelley and Arthur, is not a protein, and for that matter, not a proteinase. Further analysis is necessary to identify the active component(s) in cowhage. This substance may be a useful tool to determine the mechanism of itch induction.

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Identification of Novel Genes Involved in Cytokine-Mediated Keratinocyte Activation and Dendritic Cell Costimulation of T Lymphocytes Using an Antisense Oligonucleotide Library-Based Genomics Approach

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We have developed 96-well microtiter, cell-based assays to identify novel genes required for activation of keratinocytes (KC) and functional stimulation of T lymphocyte responsiveness by dendritic cells (DC). KC have been shown to be an important cell type in the development of psoriasis, overexpressing certain cytokines and chemokines as well as displaying abnormal proliferative behavior. In our studies, we used pooled human neonatal epidermal keratinocytes treated with potent, validated antisense oligonucleotides (ASO) that inhibit various human genes. Forty-eight hours after ASO treatment the KC are induced with TNF-α overnight and then assayed for ICAM-1 cell surface expression and IL-8 secretion by ELISA. The results of screening 80 optimized ASO inhibitors and 8 control ASO provided us with a list of 8 genes that significantly inhibit ICAM-1 and/or IL-8 expression. A primary DC-based T cell costimulation assay measuring IL-2 production was also utilized to identify several active ASO from a library of 240 separate sequences. We then performed detailed dose–response and apoptosis assays in order to determine which genes block cell activation or differentiation without inducing programmed cell death. Several genes were found to mediate both KC activation by TNF-α and DC-induced costimulation of T cells and thus represent novel therapeutic targets for psoriasis and other inflammatory skin diseases.

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SB-203347, a 14-Kilodalton, PLA_2 Inhibitor, Reduces Ear Edema, Neutrophil Influx, and Epidermal Proliferation in a Murine Phorbol 12- Myristate 13-Acetate-Induced Ear Inflammation Model

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Elevated levels of secretory PLA_2 ($sPLA_2$) have been detected in the epidermis of psoriatic patients and are associated with increased production of leukotrienes and platelet activating factor (PAF). A 14-kDa $sPLA_2$ inhibitor, SB-203347, was previously shown to block PLA_2 activity of acid extracted human neutrophil homogenates ($IC_{50} = 4.7$ µM) and inhibit release of free arachidonic acid with subsequent reduction of leukotrienes and PAF generated by A23187-stimulated human neutrophils. Previous studies *in vivo* demonstrated that SB-203347 inhibited endotoxin-induced mortality without alteration of serum TNFalevels. Here we demonstrate that type IIA PLA_2 is present in ear homogenates following treatment with Phorbol 12- Myristate 13-Acetate (PMA) by both ELISA and Ca^{2+}-dependent Sn-2 acylhydrolysis of ^{3}H-AA *E. coli* substrate. Topical treatment with SB-203347 immediately prior to epicutaneous administration of PMA dose-dependently inhibited Sn-2 acylhydrolysis of ^{3}H-AA *E. coli* substrate with an IC_{50} of 12.4 µM. Furthermore, topically administered SB-203347 inhibited ear edema, neutrophil influx, and epidermal proliferation resulting from PMA treatment. The ability of SB-203347 to inhibit both inflammation and epidermal proliferation suggests that 14 kDa $sPLA_2$ inhibitors may be efficacious in the treatment of psoriasis.

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Expression of CD44 and its Isoforms in Inflammatory Skin Diseases

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CD44 is a transmembrane glycoprotein which exists as multiple isoforms resulting from alternative splicing of 10 variant exons (v1–v10) and post-translational modifications. The main or standard CD44 form without variant exon is CD44s which has a broad cellular distribution. The isoforms of CD44 are found on cells of epithelial origin and their function is unknown. CD44s functions as the predominant cell-surface receptor for hyaluronate (HA). In a recent study we have shown that the major role of CD44 in the skin is the regulation of keratinocyte proliferation and local HA metabolism in transgenic mice with keratinocyte-specific CD44 expression defect. Recently we have also observed a decrease in epidermal CD44 expression in lichen sclerosus et atrophicus. In this study we examined the expression of CD44s and different CD44 isoforms (CD44v3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v7v8 and CD44v10) in inflammatory skin diseases such as seborrheic dermatitis (SD), acrodermatitis chronica atrophicans (ACA), drug eruption (DE), erythema multiforme (EM), toxic epidermal necrolysis (TEN) and graft vs. host disease (GVHD) by immunohistochemistry. In SD, ACA, DE and GVHD, CD44s is highly expressed in the basal and suprabasal layers of epidermis as well as in the lymphocytes of the dermal inflammatory infiltrate. However, the expression of CD44s is markedly decreased or absent in the epidermis and in the lymphocytes of the inflammatory infiltrate in EM and TEN. In the epidermis, CD44v3 is expressed in SD, ACA and DE, decreased in GVHD, and absent in EM and TEN; CD44v4 is expressed in ACA, decreased in SD, DE, EM and GVHD, and absent in TEN; CD44v5, CD44v6 and CD44v7 are expressed in SD, ACA, DE and GVHD, and absent in EM and TEN; CD44v7v8 is expressed in SD and GVHD, and absent in ACA, DE, EM and TEN; CD44v10 is expressed in SD and ACA, decreased in DE and GVHD, and absent in EM and TEN. In the lymphocytes of the inflammatory infiltrate CD44v4 is highly expressed in all diseases; CD44v6 is faintly expressed in DE, EM, TEN and GVHD, and absent in SD and ACA; CD44v7 is faintly expressed in DE, TEN and GVHD, and absent in SD, ACA and EM; CD44v10 is expressed in SD, EM, TEN and faintly in GVHD, and absent in ACA and DE; CD44v3, CD44v5 and CD44v7v8 are absent in all diseases. These results show that the expression of CD44 molecules in the epidermis and lymphocytes is differentially regulated in inflammatory skin diseases.

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Chemotaxis and Actin Polymerization of Human Dendritic Cells Depend on Rho Proteins

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Dendritic cells (DCs) are one of the principle initiators of immune responses. They are able to migrate into target sites, process antigens and activate naive T cells. The intracellular signalling cascade regulating the recruitement of DCs into target site is not well understood. However, several chemotaxins relevant for this process have previously been identified. Reorganization of the actin cytoskeleton by flow cytometry and activation of small GTPases of the Rho protein familily by prezipitation with rhotecine in human DCs after stimulation with chemotaxins such as C5a and PAF has been revealed in the present study. To show the relevance of the actin cytoskeleton reorganization in DCs Clostridium botulinum C2 toxin has been used. This toxin inhibits the migration and actin response through ADP-ribosylation of actin molecules, but did not interfere with other intracellular signalling events such as the mobilization of Ca2+ from intracellular stores. The function of Rho proteins was analyzed with the fusion toxin C2IN-C3. This exoenzyme selectively modified Rho proteins by ADP-ribosylation and blocked the actin and migration response in a concentration dependent manner. To our best knowledge we have shown for the first time that migration and reorganization of the actin cytoskeleton of dendritic cells is regulated by Rho GTPases.

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Blood Levels of Soluble Vascular Adhesion Molecules, VCAM-1 and E-Selectin, are Increased in Clinically Amyopathic Dermatomyositis

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Patients with classical dermatomyositis (CDM) express VCAM-1 and E-selectin in the microvessels of inflamed muscular and cutaneous tissue. In addition, patients with CDM have recently been reported to display elevated blood levels of VCAM-1 and E-selectin. Whether these soluble vascular adhesion molecules reflect activation of the muscular or dermal microvasculature (or both) has not been determined. To examine the component of soluble adhesion molecules contributed by inflammation predominately involving the dermal microvasculature, we measured levels of soluble VCAM-1 and E-selectin in the serum of 12 clinically amyopathic DM patients (C-ADM) (i.e. patients having the hallmark cutaneous manifestations of DM for 6 months or longed without clinically significant evidence of inflammatory myositis [i.e. No proximal muscular weakness and normal muscle enzymes]. In addition, 17 CDM patients were also studied for comparison. Stored patient and control serum samples were analyzed by ELISA. Optical density values greater than 3 s.e.m. Above the mean produced by normal control sera were considered abnormal. Elevated levels of VCAM-1 were observed in 9/17 (53%) CDM and 5/12 (42%) C-ADM patients ($p = 0.8845$ by chi square). Elevated E-selectin levels were found in 3/17 (18%) CDM and 9/12 (75%) C-ADM patients ($p = 0.0077$ by chi square). The presence of similar rates of elevation of soluble VCAM-1 in CDM and C-ADM patients suggests that circulating levels of this vascular adhesion molecule could reflect activation of the muscular or dermal microvasculature (or both). The much higher rate of elevated soluble E-selectin levels in C-ADM patients suggests that circulating levels of this vascular adhesion molecule might better reflect (or be limited to) activation of the dermal microvasculature. In addition, if soluble E-selectin levels can be shown to correlate positively with the extent of clinical cutaneous inflammation, soluble E-selectin levels could represent a quantifiable surrogate marker of the degree of cutaneous vascular inflammation in CDM and C-ADM.

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Discovery of Novel JAK Kinase Inhibitors with Cell-Based Assays

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Several observations indicate that keratinocyte-derived IL-7 supports the viability and modifies the behavior of T cells within the skin. Therefore the IL-7 signaling pathway may be a useful pharmacological target for the treatment of inflammatory skin disease. Tyrphostins are a class of chemical compounds that block cytokine signal transduction by inhibiting the Janus family of cellular kinases (JAK). The most potent of these, AG490 or B42, blocks the responses of various cells to a number of cytokines by inhibiting both JAK2 and JAK3 kinases. Cellular responses to IL-3, –5, –6, –12, IFN-gamma and GM-CSF depend upon JAK2 while responses to IL-2, –4, –7, –9 and –15 require JAK3. By inhibiting both of these kinases, AG490 exerts broad suppressive effects on the immune system. In an effort to identify compounds that are more selective and potent inhibitors of the JAK kinases a library of 600 distinct derivatives of AG490 were synthesized using a combinatorial approach involving radiofrequency tagging and directed sorting of solid phase reactors. Simple cell proliferation assays were used to evaluate the effectiveness of each of these compounds as inhibitors of JAK2 and JAK3. The IL-3 dependent Baf3 cell line was used to measure the inhibition of JAK2 and the IL-7 dependent 2E8 cell line was used to measure the inhibition of JAK3. Two closely related compounds were identified which are approximately 8 times more potent inhibitors of JAK3 and one of these is a significantly less potent inhibitor of JAK2. This may limit the inhibitory effects of these compounds to T cells and reduce their effects on other cells, thereby minimizing immunosuppression.

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Enhanced Contact Hypersensitivity in TP-Deficient Mice: Implication of TXA2 as an Essential Negative Feedback Regulator in Skin Immune Response

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Thromboxane A2 (TXA2) is a potent stimulator of platelets and constriction of vascular smooth muscle. However, the TXA2 receptor (TP) mRNA is abundant in lymphocytes and the TX synthase is especially abundant in cells of macrophage lineage, which suggests that TXA2 plays an important role in immune system. To ascertain the essential functions of TXA2, we generated and characterized the TP-deficient mice, which exhibit an enhanced (about two fold) and prolonged contact hypersensitivity (CHS) response elicited with DNFB. This phenotype was pharmacologically reproduced by TP antagonist administration during the sensitization phase but not during the challenge phase, indicating that TXA2-TP functions at the sensitization phase. In CHS model, the number of cervical lymphocytes, especially Th1 cytokine producing cells, of TP-deficient mice was significantly larger than that of WT mice. But TP-deficient mice do not have defects in Langerhans cell function, such as migration and costimulatory molecule expression. Therefore we examined the interaction of APC-T cell response *in vitro*. DNBS induced T cell proliferation of DNFB sensitized lymph node cells of TP-deficient mice was more than that of WT mice. The purified naive CD4 cell proliferation induced by con A with APC was significantly suppressed (40%) by a TP agonist. This phenomenon was seen in T cells from WT mice but not from TP-deficient mice and the suppression in WT cells was observed irrespective of the origin of cocultured APCs. In this model, TP agonist affected neither the IL-2 production nor the receptor expression. On the other hand, TP agonist significantly suppressed the proliferation by the addition of exogenous IL-2. These findings indicate that TXA2 acts as a negative feedback regulator on skin inflammatory response on T cells but not on APCs, by suppressing the IL-2 responses during sensitization phase.

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Protective Role Played by CD39 on Langerhans Cells Against Chemically Induced Skin Inflammation

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Although ecto-ATPase/ADPase activities have been widely used 30 years as useful markers of epidermal Langerhans cells (LC), the molecular identity or physiological function of these activities remain unknown. CD39, which was originally described as an obscure activation marker expressed by B cells, dendritic cells, and endothelial cells, has been recently identified as a vascular ecto-ATPase/ADPase, now termed collectively as ecto-nucleoside triphosphate diphosphohydrolase (NTPDase). We detected CD39 mRNA and/or protein expression almost exclusively within the IA^+ population in mouse epidermis and in LC-derived XS52 and XS106 lines, but not in keratinocyte or epidermal γδ T cell lines. Moreover, CD39-deficient mice showed normal surface densities of $IA^+/DEC205^+$ LC, whereas these $CD39^{-/-}$ LC lacked apparent ecto-NTPDase activities. Thus, we conclude that CD39 alone is responsible for LC-associated ecto-NTPDase activities. With respect to function, $CD39^{-/-}$ mice developed significantly ($p < 0.01$, 10 mice per panel) exacerbated ear swelling responses to topically applied croton oil, benzalkonium chloride (BAC), and ethyl phenylpropiolate (EPP) than did $CD39^{+/+}$ mice in terms of both the magnitude and time course. Late phase responses observed in $CD39^{-/-}$ mice were characterized histologically by striking epidermal hyperplasia and marked leukocyte infiltration. $CD39^{+/-}$ mice showed intermediate responses between the two panels. Interestingly, inflammatory responses to the fourth irritant chemical, phenol, were comparable between $CD39^{-/-}$ mice and $CD39^{+/+}$ mice. Local administration of soluble NTPDase reduced the extent of croton oil-induced ear swelling in $CD39^{-/-}$ mice to the normal level, supporting the anti-inflammatory role for CD39. Upon exposure to croton oil, BAC or EPP, but not to phenol, Pam 212 keratinocytes rapidly (< 10 min) released biologically relevant amounts of ATP into culture media. Addition of CD39-expressing XS52 cells in keratinocyte cultures significantly reduced ATP levels in the media. Thus, we conclude that LC-associated CD39 plays a protective role by hydrolyzing otherwise pro-inflammatory ATP that is released by neighboring keratinocytes in response to selected forms of environmental stimuli. Our results reveal two distinct (i.e. ATP-dependent and -independent) pathogenic mechanisms of irritant contact dermatitis and form the basis to develop an *in vitro* screening system that can be used to identify causative chemicals.

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SDZ ASM 981 Potently Inhibits the Induction of Coreceptors Involved in the Accessory Cell-Dependent Activation of Inflammation-Mediating T Cells

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Previously, we have reported that SDZ ASM 981 is a potent inhibitor of inflammatory cytokine synthesis in both T cells and mast cells *in vitro* and inhibits the allergic contact hypersensitivity (ACH) reaction *in vivo*. As the ACH reaction is dependent on efficient priming of responder T cells by antigen-presenting cells, we studied whether SDZ ASM 981 would also interfere with the activation-associated up-regulation of coreceptor molecules on helper T cells. Particularly, we focussed on coreceptors recently proposed to be involved in the activation and expansion of effector T cells in autoimmunity such as CD134 (OX-40), and CD137 (4–1BB) previously suggested to mediate preferential activation of CD8+ T cells and Th1 cells producing inflammatory cytokines. For comparison, we included the activation markers CD54 (ICAM-1) and CD25 (high affinity IL-2 receptor). To mimic the physiological situation as closely as possible, we have used human monocyte-derived dendritic cells (MoDC) as potent stimulators of highly purified CD4+ T cells in the allogeneic mixed lymphocyte reaction (allo-MLR). The allo-MLR was performed in the presence and absence of SDZ ASM 981 and Cyclosporin A (CyA) using a 50-fold excess of T cells over MoDC. After three days, the level of expression of the T cell coreceptors was determined by flow cytometry. SDZ ASM 981 dose-dependently inhibited the up-regulation of CD25, CD54, CD134 and CD137 with 80% inhibition reached at about 10 nM. In this respect, SDZ ASM 981 was at least 10-fold more potent than CyA. These differences in the relative potency of the compounds were also revealed for the inhibition of T cell proliferation in allo-MLR as determined by parallel thymidine incorporation assays. Taken together, SDZ ASM 981 not only inhibits T cell proliferation by blockade of cytokine synthesis but also prevents the up-regulation of coreceptors thought to be critical for the development of inflammatory effector T cells.

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Topical PPAR-α Activators Display Anti-Inflammatory Activity in Irritant and Allergic Contact Dermatitis Models

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Activators of PPAR-α, a nuclear hormone receptor that heterodimerizes with RXR, stimulate epidermal differentiation and inhibit proliferation. Here, we describe the anti-inflammatory effect of PPAR-α activators in irritant and allergic contact dermatitis induced in mouse ears with TPA and oxazalone, respectively. The PPAR-α activators were applied 45 min and 4 h after TPA or oxazalone treatment. Ear thickness and weight were measured at 18 h, and assessed by histology and immunohistochemistry for TNF-α and IL-1α. Clofibrate (0.02%) reduced the TPA-induced increase in ear thickness and weight by ~50% (both $p < 0.01$), vs. vehicle- and stearic acid-(not a PPAR-α activator)-treated controls. Two other PPAR-α activators, WY 14643 (0.03%) and linoleic acid (0.001%), produced similar results and all were comparable to clobetasol 0.05%. Histology of clofibrate + TPA-treated ears revealed a marked reduction in TPA-induced inflammation, and immunohistochemistry showed decreased TNF-α and IL-1α levels throughout the epidermis. Since clofibrate did not significantly reduce TPA-induced ear swelling in PPAR-α knockout animals, the anti-inflammatory activity is receptor-mediated. Finally, PPAR-α activators also reduced oxazolone-induced ear thickness and ear weight by 50%–55% ($p < 0.05$). These studies demonstrate that topically applied PPAR-α activators possess receptor-mediated, anti-inflammatory activity in both irritant and allergic contact dermatitis models. The anti-inflammatory activity of these compounds, coupled with their antimitogenic and prodifferentiating effects, suggest that they could prove beneficial for the treatment of cutaneous inflammatory dermatoses.

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Oxysterol Ligands of LXR Display Anti-Inflammatory Activities in Irritant and Allergic Contact Dermatitis Models

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Recently, we have shown that oxysterols stimulate epidermal differentiation and development, and inhibit keratinocyte proliferation by activating liver X receptors (LXR) that are present in keratinocytes. LXR α and β are members of the nuclear hormone family that heterodimerize with RXR to regulate gene expression. In the present study, the anti-inflammatory effect of two oxysterols, 22(R)-hydroxycholesterol (22ROH) and 25-hydroxycholesterol (25OH), were examined utilizing models of irritant and allergic contact dermatitis. Irritant dermatitis was induced by applying TPA to the ears of CD1 mice followed by treatment with vehicle alone (acetone), 22ROH, 25OH, cholesterol, or clobetasol 45 min and 4 h following TPA. Anti-inflammatory effects were determined 18 h after TPA by measuring ear thickness and weight (6 mm punch biopsy). Whereas TPA alone induced a ~2-fold increase in ear weight and thickness, both 22ROH and 25OH treatment significantly reversed this increase (50% decrease in thickness; 60–75% decrease in weight), comparable to anti-inflammatory effects observed with topical clobetasol treatment. Histology also revealed a marked decrease in dermal inflammation in oxysterol-, but not vehicle- or cholesterol-treated animals. Immunohistochemistry also demonstrated a decline in the production of the proinflammatory cytokines, IL-1α and TNFα, by oxysterol treatment. Since topical cholesterol did not affect TPA-induced inflammation, the inhibition by oxysterols can not be attributed to a nonspecific sterol effect. Finally, both 22ROH and 25OH also reduced ear thickness/weight by ~40% in the oxazolone allergic contact dermatitis model. These studies demonstrate that naturally occurring oxysterol ligands of LXR display anti-inflammatory activity in both irritant and allergic contact dermatitis models. Oxysterols could provide a new class of therapeutic agents for the treatment of cutaneous inflammatory disorders.

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PTEN WAP–/– Mice, a Model of Spontaneous Cutaneous Inflammation

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PTEN is a tumor suppressor with sequence homology to the protein tyrosine phosphatases and the cytoskeletal protein tensin. The PTEN gene is the most highly mutated tumor suppressor gene in post-p53 era. Somatic deletions or mutations of this gene have identified in a wide range of tumors. PTEN plays a significant role not only in inducing programming apoptosis and cell cycle arrest, but also in other aspects of cell physiology, including the regulation of cell adhesion, migration and differentiation. In addition, *in vitro* studies suggest that PTEN may participate in the regulation of T cell survival and T cell receptor signaling. Recently, we have developed tissue-specific PTEN knockout mice (PTEN WAP–/–) with PTEN deficiency in the skin. Intriguingly, PTEN WAP–/– mice spontaneously developed chronic cutaneous inflammation. Clinically, the dermatitis was characterized by thickened and reddened skin, and abnormal hair. The dermatitis occurred on the trunk, head and ears. Histological examination of the skin sections revealed marked hyperkeratosis, acanthosis, dermal thickening and mixed cellular infiltrate composed of mononuclear cells and neutrophils, hyperplasia of the pilosebaceous units and follicular plugging, and neovascularization in PTEN WAP–/– mice, compared to the normal skin in PTEN WAP+/– littermates. Microscopically, the hair of PTEN WAP–/– mice showed abnormality of the shape, size and pigmentation pattern. The dermatitis observed in PTEN-deficient mice had similarities with T-cell-dependent chronic inflammatory skin diseases such as psoriasis. The spontaneous occurrence of dermatitis in PTEN-deficient mice suggests that PTEN may be involved in cutaneous immune and inflammatory responses. PTEN WAP–/– mice provide for an experimental model of cutaneous immune and inflammatory dermatoses.

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Attenuation of GAP-43 and NF-200 Innervation in Chronic Venous Ulceration

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Clinical and electrophysiological evidence of peripheral neuropathy and impairment of C-fibre function have been found in venous ulcer patients. We have used immunohistochemistry and image analysis quantification of Growth-Associated Protein 43 (GAP-43) and Neurofilament-200 (NF-200) immunoreactive (IR) nerve fibres to examine the distribution of peptidergic and sensory nerves in the skin adjacent to venous ulcers. Punch biopsies from patients with ulcers which healed within 3 months ($n = 14$) and nonhealing ulcers ($n = 15$) were compared with age- and site-matched control subjects ($n = 12$). Total GAP-43 IR fibre length was significantly decreased in the dermis and epidermis in both patient groups (Mann–Whitney U test $p < 0.001$ in all cases). Dermis *controls*, median 1795 µm (IQR 743 µm-3190 µm); *healers*, 198 (4–366); *nonhealers*, 197 (41–239); epidermis: *controls* 153 (65–304); *healers*, 0 (0–22); *nonhealers*, 0 (0–0). Total NF-200 IR fibre length was decreased in the dermis in *nonhealers* only (Mann–Whitney U test $p = 0.008$): *controls*, 2071 (1215–3177); *nonhealers*, 581 (327–1681). Total epidermal fibre number, but not length was slightly reduced in both patient groups (Mann–Whitney U tests $p = 0.039$ *healers*, $p = 0.028$ *nonhealers*): *controls*, 4 (0–10); *healers*, 0 (0–2); *nonhealers*, 0 (0–1). Many GAP-43 fibres have been shown to contain peptidergic neurotransmitters such as calcitonin gene-related peptide and substance P. Depletion of the cutaneous peptidergic innervation will affect a number of structures and processes that may impact on tissue integrity and healing. Sensory nerve fibres (indicated by NF-200 IR) appear to be depleted to some extent in these patients. This could result in decreased ability to respond to minor skin injury that may in turn lead to ulcer development.

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Neutral Endopeptidase Inhibition Improves Wound Repair in Diabetic Mice

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In response to injury, C-fiber sensory nerves contribute to inflammation through release of substance P (SP). Neutral endopeptidase (NEP) regulates SP effects through enzymatic degradation. Skin and ulcers from patients with diabetes show increased NEP localization and activity. We hypothesized that NEP activity would be increased in diabetic (db/db) mice and NEP inhibition would improve inflammation and closure kinetics in excisional wounds. NEP activity of skin from db/db mice and db/– littermates was determined fluorometrically. Using a 6-mm biopsy punch, a dorsal excisional wound was created on the mice. Wounds were covered with a semiocclusive dressing and treated for 7 days with normal saline (NS) or thiorphan. In one experiment, mice were followed with weekly photographs until the wounds appeared closed. In another experiment, wounds were harvested at 7 time points for H&E analysis. NEP activity in unwounded db/db skin exceeded activity in db/– skin (20.6 vs. 7.9 pmol MNA per h per µg; $p = 0.02$). In db/db mice, thiorphan-treated wounds healed faster (18d) than NS-treated (24d) wounds ($p < 0.05$). There was no difference in the size of healed wounds suggesting that thiorphan did not affect contraction. Analysis of wound histology using a semiquantitative scoring system suggested that inflammation was increased at 6, 24, 48 and 72 h in wounds treated with thiorphan compared to NS ($p = ns$). Increased NEP activity in skin from db/db mice correlates with NEP activity in skin from patients with diabetes and corroborates use of db/db mice to study the role of substance P after injury. NEP inhibition improved wound closure kinetics in db/db mice without affecting contraction suggesting that epithelialization may be augmented. This improvement may be due to increased availability of substance P and enhanced inflammation in the early stages of wound healing.

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Selective COX-2 Inhibition Does Not Alter Healing of Incisional Sutured Skin Wounds in SKH1 Mice

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Using an incisional wound model in SKH1 hairless mice, we evaluated the role of cyclooxygenase-2 (COX-2) in the wound healing process by assessing the effects of therapeutic and supra-therapeutic doses of nonselective COX inhibitors (naproxen and diclofenac), a selective COX-2 inhibitor (SC-791), and dexamethasone. Mice were administered vehicle or drugs 2–6 days prior to wounding. A 1.5-cm incision was made on the dorsum of mice and closed with three equidistant 5–0 nylon sutures. Wound healing was monitored histologically, wound strength was evaluated using a tensiometer and COX-2 protein expression was assessed by Western blot analysis and immunohistochemistry up to 28 days postincision. Both the selective COX-2 and nonselective COX inhibitors at therapeutic doses had no significant effect on the macroscopic and microscopic morphology of the wounds, whereas dexamethasone treatment resulted in epidermal and adnexal atrophy. COX-2 expression was observed over the first week postincision peaking at day 3 in the presence and absence of both the selective and nonselective COX-2 inhibitors. Infiltrating macrophages, as well as basal and migrating keratinocytes, expressed COX-2 and this pattern of expression was not affected by therapeutic drug treatment. In addition, selective and nonselective COX-2 inhibitors did not alter wound strength in contrast to dexamethasone, which reduced the tensile strength of the healing wound by up to 38%. In conclusion, these data indicate that selective COX-2 inhibition does not dramatically affect wound healing. These data suggest that COX-2 may not play a significant biological role in skin wound healing or redundant pathways can compensate for inhibition of COX-2.

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Increased E-Selectin, IL-8 and IL-10 Gene Expression in Human Skin After Minimal Trauma: A Potential Explanation of Regional Distribution of Skin Lesions

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Although dermatitis herpetiformis (DH) is characterized by the distribution of skin lesions on extensor surfaces such as elbows, knees and buttocks, the mechanism of this localization is not known. We have demonstrated that neutrophils in patients with DH have an increased expression of neutrophil CD11b, however, without appropriate expression of endothelial cell adhesion molecules, neutrophil extravasation can not occur and skin lesions will not develop. We postulated that minor trauma on extensor surfaces may lead to the expression of adhesion molecules, resulting in the development of skin lesions in these areas of minimal trauma. In order to test this hypothesis we gently rubbed normal inner arm skin of three subjects for 2 min with a pencil eraser. After 4 h skin biopsies were obtained from the rubbed site and from adjacent normal, inner arm skin. Total RNA was immediately isolated from the skin biopsies and cDNA produced by reverse transcriptase. Real Time, Polymerase Chain Reactions (PCR) were performed using primers for E-selectin and the cytokines IL-1α, –1β, –2, –4, –5, –8, –10, –12p35, –12p40, –15, IFN-γ and TNF-α. Expression was normalized to the level of 18sRNA and gene expression was compared between normal inner arm skin and rubbed arm skin for each subject. After 4 h a barely perceptible erythema was seen with no other changes noted in the rubbed skin. Real Time-PCR analysis revealed a marked increase in expression of E-selectin in the rubbed skin when compared to E-selectin expression in normal, unrubbed inner arm skin (79.3; ratio mean rubbed skin expression/normal skin expression). A similar increased expression of IL-8 (85.9) and IL-10 (38.1) was seen. No significant difference in the expression of the other cytokines was noted (0.69–3.3; ratio mean rubbed skin expression/normal skin expression). These findings suggest that minor trauma to skin may induce specific expression of E-selectin, IL-8 and IL-10, which in the presence of partially activated inflammatory cells would enhance extravasation of neutrophils and lead to localized development of skin lesions. The localized increased expression of adhesion molecules and cytokines in the skin after minor trauma may play an important role in regional expression of skin lesions and clinical observations such as the Koebner phenomena.

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Mast Cells and TNFα Contribute to Inflammatory Cell Recruitment to Sites of Early Skin Granuloma Formation

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The induction of skin granuloma (SG)-formation, e.g. in foreign body granuloma or cutaneous lesions of leishmaniasis, is characterized by the influx of neutrophils (PMN) and macrophages. However, little is known about how these cells are recruited to the sites of inflammation. Using a murine model of polyacrylamide gel (PAG)-induced development of SG, we have characterized the role of mast cells (MC) and TNFa in the early stages of SG-formation. Genetically MC-deficient Kit^W/Kit^{W-v}-mice and normal +/+ mice were injected subcutaneously with PAG (Biogel P-100, 1 ml, back skin) and infiltrating cells were recovered from the granulomas for FACS analysis. PAG-induced granulomas in Kit^W/Kit^{W-v}-mice contained markedly less infiltrating cells as compared to +/+ mice at all time points studied: Kit^W/Kit^{W-v}-mice = 0.3, 2, and 17 vs. +/+ mice = 1, 19, and 34×10^6 cells/granuloma at 6 h, 12 h, and 24 h, respectively ($p < 0.001$). In both genotypes, 90% of the cells recovered from the granulomas at all time points reported were identified as PMN by positive staining for antineutrophil mAb (7/4 and Gr-1). In +/+ mice, numbers of degranulated MC at sites of SG-formation were significantly increased 6 h after injection of PAG as compared to vehicle-injected control skin ($p < 0.05$), indicating that PMN recruitment to sites of SG-formation is mediated, at least in part, by activated skin MC. Since MC produce, store, and release proinflammatory TNFα, a potent PMN chemoattractant, we have assessed SG-formation in TNFα-deficient (TNFα−/−)-mice and wild type (WT) mice. Notably, 50-fold more cells were recovered from granulomas in WT mice as compared to those in TNFα−/− mice (57 vs. 1×10^6 cells/granuloma at 24 h, $p < 0.001$). These data suggest that MC and TNFα play a key role in SG-formation in mice.

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Myeloperoxidase-Deficient Mice are Resistant to Experimental Bullous Pemphigoid

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Subepidermal blistering in experimental Bullous pemphigoid (BP) is triggered by anti-BP180 antibodies and depends on complement activation, mast cell degranulation, and neutrophil infiltration. We have recently shown that mice deficient in gelatinase B (GB) and neutrophil elastase (NE) are resistant to experimental BP. Our further studies reveal that gelatinase B contributes to tissue damage indirectly by proteolytically inactivating α1-proteinase inhibitor (α1-PI) to release neutrophil elastase, which directly causes tissue injury in experimental BP. Since reactive oxygen species (ROS) generated through myeloperoxidase (MPO) pathway also can inactivate α1-PI *in vitro*, in this study we investigated the role of ROS in experimental BP using mice lacking MPO. Pathogenic anti-BP180 IgG induced subepidermal blisters in wild-type mice but not MPO-deficient mice. Further, intradermal injection of the MPO inhibitor or scavenger abolished blistering in wild-type mice triggered by pathogenic IgG. Western blot assay reveals significantly higher levels of α1-PI in the skin of MPO-deficient mice than wild-type controls at the IgG-injected sites. These results suggest that ROS, in concert with gelatinase B, inactivate α1-PI and the unchecked NE damages tissue at the dermal-epidermal junction in BP.

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BP180 (BPAG2) is a Neutrophil Chemoattractant *In Vitro* and *In Vivo* After Proteolysis by Neutrophil Elastase

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Bullous pemphigoid (BP) is an autoimmune disease characterized by subepidermal blisters and autoantibodies against two hemidesmosomal proteins, BP230 (BPAG1) and BP180 (BPAG2). Passive transfer of anti-BP180 IgG into neonatal mice induces BP-like lesions by complement activation and neutrophil (PMN) infiltration. We have recently shown that neutrophil elastase, gelatinase B and α1-proteinase inhibitor (α1-PI) are key mediators involved in BP blister formation. The purpose of this study was to search for additional PMN chemotactic factors that may be relevant in modulating the lesions of BP. We tested whether NE-derived fragments of BP180 recruit inflammatory cells. Recombinant murine BP180 (mBP180ABC) was digested by NE and the proteolytic fragments (mBP180–18 kDa, mBP180–15 kDa, and mBP180–9 kDa) were purified and assayed for PMN chemotactic activity *in vitro* using a modified-Boyden chamber and murine PMN. Pro-inflammatory activity *in vivo* was tested by subcutaneous injection of these BP180 fragments into mice. Biopsies of the injection site at different time points were evaluated. PMN showed chemotaxis to mBP180–15 kDa at the peak response at 10(–7) M, which exceeded the maximum response to the positive control, fMLP at 10(–4) M. Fragments mBP180–18 kDa and mBP180–9 kDa showed no chemotactic activity. Likewise, injected mBP180–15 kDa induced an early PMN infiltrate and a late eosinophilic infiltrate of the injected sites. Fragments mBP180–18 kDa and mBP180–9 kDa did not elicit inflammation *in vivo*. These results indicate that cleavage of BP-180 by NE releases BP180 peptides that are strongly chemotactic of PMNs and suggest that inflammatory injury in BP may result in part, by release of proinflammatory factors derived from hemidesmosomal proteins.

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Regulation of Tissue Factor in Dermal Endothelial Cells

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Inflammation is accompanied by activation of the coagulation cascade, manifested by thrombosis and fibrin generation. While endothelial cells normally provide a nonthrombogenic surface, inflammatory mediators may induce the expression of tissue factor (TF), rendering their surface thrombogenic. In order to define the mechanisms regulating the expression of TF in the skin microvasculature, we examined TF expression in human dermal microvascular endothelial cells (HDMEC). Quiescent HDMEC did not constitutively express TF protein or mRNA, but could be induced to express TF by treatment with TNF, IL-1, LPS, or PMA. Treatment with serum induced modest expression of TF and augmented TNF-mediated TF induction. In order to define the pathways mediating TF induction, we examined the effects of MG-132, an inhibitor of NF-κB activation, and SB203580 an inhibitor of p38/JNK activation. Despite an almost complete inhibition of TNF-induced E-selectin expression, pretreatment with MG132 only partially blocked TNF-induced TF induction. In contrast, SB203580 almost completely inhibited TNF-induced TF expression but only partially inhibited TNF-mediated E-selectin induction. Neither MG-132 nor SB203580 inhibited serum-induced TF expression. These data suggest mediators that induce TF in HDMEC may utilize multiple pathways and that serum induction of TF occurs via an NF-κB, p38/JNK independent pathway. In order to define the potential mediators in serum, we examined the effects of the platelet derived growth factors PDGF and basic FGF as well as the lipid mediator sphingosine-1 phosphate (S1P). S1P-induced dose-dependent increases in TF expression, while PDGF and bFGF had no effect, suggesting the serum effects on TF expression may be mediated in part by this platelet-derived lipid. In conclusion, these data suggest that interaction between pro-inflammatory cytokines and platelet-derived lipid mediators may play a role in the expression of TF in the dermal vasculature.

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Fibrinogen Derivatives Control a Novel Pathway of Transendothelial Leukocyte Traffic

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The conversion of fibrinogen into its biologically active derivatives is tightly regulated and usually limited to certain segments of the vascular bed. It occurs through proteolytic cleavage of fibrinopeptides by, e.g. thrombin or urokinase-type plasminogen activator (uPA), resulting in the exposure of a neo N-terminus of the Aa- and/or Bb-chains of fibrinogen. To analyze a putative role of these neo N-termini in inflammation, we used a truncated form of fibrinogen called N-terminal disulfide knot of fibrinogen (NDSK), which is unable to polymerize and created by cyanobromide digestion. To quantify inflammation, we employed two models, first SCID mice engrafted with human skin and human lymphocytes, second an *in vitro* transmigration assay. We show that thrombin-treated NDSK (lacking fibrinopeptides A and B) caused vigorous transmigration of monocytes and lymphocytes across endothelial barriers. Transmigration depended on the interaction of the neo N-terminus of the Bb-chain of fibrinogen with endothelial VE-cadherin and was blocked by peptide Bb15–42. Monocytes use CD11c to attach to the neo N-terminus of the Aa-chain. In contrast to monocytes, lymphocyte transmigration was independent from cleavage of fibrinopeptide A. It was thus not blocked by peptide Aa17–19 and also functioned with uPA-treated NDSK (lacking fibrinopeptide B only). In conclusion, we describe selective functions of two different proteolytic fibrinogen derivatives in monocyte and lymphocyte transmigration. By bridging leukocytes with the junctional protein VE-cadherin, they mimic PECAM-1-like functions.

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Different Interaction of Mast Cells with Human Endothelial Cells and Fibroblasts

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In a number of chronic inflammatory conditions resulting into fibrosis, perivascular mononuclear cell infiltration including mast cells (MC) have been shown before the onset of vascular injury and interstitial fibrosis. These observations suggest a role for MC to induce endothelial cell (EC) injury and fibroblast (FB) proliferation and collagen synthesis. In view of these observations, the interactions of MC to EC and FB were studied. MC adhesion to EC and FB showed time-dependent increase reaching plateau at 1 and 3 h, respectively. Added MC, the proliferation of EC showed dose dependent decrease, but that of FB dose dependent increase. MC, MC supernatant and sonicated MC induced dose dependent cytotoxic activity to EC, which cytotoxicity was inhibited by trypsin inhibitor. FB cocultured with MC showed 9.95 times collagen synthesis and 11.0 times protein synthesis compared with FB without MC. These results showed that (1) MC attached to EC, inhibited the proliferation by cytotoxic activity to EC, which was due to a kind of proteolytic enzyme involving trypsin, (2) MC had proliferative and collagen synthetic activity to FB. These results suggest a possible role of MC to participate in a number of chronic inflammatory diseases resulting into vascular injury and interstitial fibrosis.

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Activated Macrophages Express Scavenger Receptors in Skin of Mice with Scleredermatous Graft *vs*. Host Disease, a Model for Human Scleroderma

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Murine sclerodermatous graft vs. host disease (Scl GVHD) is a model for human scleroderma in which skin thickening, lung fibrosis, and cutaneous TGF-β1 and collagen mRNA up-regulation occur by day 21 post bone marrow transplantation (BMT). Skin fibrosis, detectable by day 14 post-BMT in Scl GVHD, may be monocyte/macrophage (Mo/Mph) driven as these are the predominant and earliest cells in cutaneous inflammation. We characterized cutaneous immune cells in murine Scl GVHD and examined their activation status. We produced Scl GVHD by transplanting lethally irradiated female BALB/c mice ($H\text{-}2^d$) with bone marrow and spleen cells from male B10.D2 mice ($H\text{-}2^d$). Syngeneic transplanted BALB/c mice do not develop disease. A significant decrease in the numbers of intact mast cells in sections of Scl GVHD skin from days 7–21 reflects early activation and degranulation of mast cells, previously shown by others in GVHD and scleroderma. By FACS analysis of dermal cell suspensions and by immunostaining, cutaneous CD11b+ cell infiltrates-primarily Mo/Mphs-predominate over T cells on days 14 and 21. PCR analysis of Y chromosome sequences on DNA extracted from female Scl GVHD mice suggest that infiltrating cells are of male donor origin. Activation markers $I\text{-}A^d$ (MHC class II alloantigen), CD11b, and 2F8 and Marco macrophage scavenger receptors are up-regulated 3–6-fold in Scl GVHD vs. controls, suggesting that in early fibrosing disease, macrophages in skin are activated and may actively present antigens. Mice were given 2 doses of 150 mg of neutralizing antipan TGF-β Abs or control rabbit IgG intravenously on days 1 and 6 post-BMT. Abs to TGF-β prevent macrophage influx as well as up-regulation of macrophage activation markers 2F8 and I-A, and abrogate skin thickening. Therefore, activated macrophages may be a potential target for therapy of scleroderma and other fibrosing disorders.

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Cutaneous TGF-β and C-C Chemokines in Murine Sclerodermatous Graft *vs*. Host Disease (Scl GVHD), a Model for Human Scleroderma

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Murine Scl GVHD is a model for human scleroderma, with skin thickening, lung fibrosis, and up-regulation of cutaneous TGF-β1 and type I collagen mRNAs occurring by day 21 post bone marrow transplantation (BMT). Fibrosis in Scl GVHD may be driven by infiltrating TGF-β1-producing monocyte/macrophages (Mo/Mph) and T cells, and their recruitment may be C-C chemokine potentiated. We characterized the cutaneous chemokine environment, infiltrating immune cell types, and the efficacy of anti-TGF-β Ab in preventing skin thickening in the model. We produced Scl GVHD by transplanting lethally irradiated BALB/c ($H\text{-}2^d$) mice with B10.D2 ($H\text{-}2^d$) bone marrow and spleen cells. Syngeneic transplanted BALB/c mice served as controls. Mice were administered 2 doses of 150 mg of antipan TGF-β Abs or control rabbit IgG on days 1 and 6 post-BMT. Skin thickening is detectable by day 14, and skin is more than 40% thicker by day 21 in Scl GVHD but not control animals. C-C chemokines MCP-1, MIP-1a, and RANTES mRNAs are elevated in Scl GVHD vs. control mice by day 7 and remain elevated throughout the early disease process. By FACS analysis, on day 14 cutaneous CD11b+ cell infiltrates are primarily Mo/Mphs that are increased 4-fold (15–30% of total skin cells). CD3+ T cells are increased more than 4-fold (5–8% of total skin cells). By RT/PCR analysis, Mo/Mphs are the main cutaneous cells producing increased TGF-β1 but not -β2 or -β3. Most importantly, we demonstrate that Abs to TGF-β prevent Scl GVHD by effectively blocking the influx of Mo/Mphs and T cells into skin, and by abrogating up-regulation of TGF-β1, thereby preventing collagen synthesis. Therefore, TGF-β1-producing mononuclear cells may be critical effector cells, and C-C chemokines may play important roles in early Scl GVHD. The Scl GVHD model is useful for testing new interventions in early fibrosing diseases, and chemokines may be new potential targets for treatment of scleroderma.

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Withdrawn

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Nerve Growth Factor Receptor Inhibitor, K252a Improves Psoriasis: Role of NGF and its Receptor System in Psoriasis

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The peripheral nervous system, in addition to sensory and motor functions can regulate an inflammatory response. The role of neurogenic inflammation in the pathogenesis of several inflammatory diseases such as psoriasis, asthma, rheumatoid arthritis, ulcerative colitis, and atopic dermatitis is currently an active field of study. Nerve growth factor (NGF) and its receptor (NGF-R) have been extensively studied in psoriasis. Increased levels of NGF in the keratinocytes and up-regulation of NGF-R in the cutaneous nerves of psoriatic plaques have been documented by several studies. Certain unique features of psoriasis substantiates an important role of NGF and its receptor system in its pathogenesis: hyperproliferation of terminal cutaneous nerves, up-regulation of neuropeptides, and resolution of active lesions of psoriasis at sites of anesthesia. In this double-blinded, placebo controlled study, we addressed the role of NGF/NGF-R system in the pathogenesis of psoriasis in an *in vivo* system using the severe combined immunodeficient (SCID) mouse model of psoriasis. The 12 transplanted psoriatic plaques on the SCID mice were treated with K252a, a high affinity NGF receptor blocker. Complete resolution of psoriasis was observed after completion of intralesional therapy with K252a at a dose of 100 microgram per kg per day for two weeks. The length of the rete pegs changed from 308.57 ± 138.72 mm to 164.64 ± 64.78 mm ($p < 0.01$, student t-test). In the eight controls (normal saline recipients) the rete peg lengths before and after therapy were 269.37 ± 57.78 and 209.37 ± 74.00 ($p > 0.1$, student t test). HLA-DR positive lymphocytic infiltrates and intraepidermal CD8 positive lymphocytes were significantly reduced in the plaques treated with K252a. We also demonstrated that K252a inhibited NGF induced mRNA and protein levels of RANTES, IFN-γ and TNF-α involved in the pathogenesis of psoriasis. This study yields direct evidence for the role of NGF/NGF-R system in the pathogenesis of psoriasis and provides insights to develop novel therapies.

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CD80/86 Antisense Oligonucleotide Therapy Improves Psoriasis: Study in the SCID Mouse Model

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Co-stimulatory molecules are essential for the activation of the T cells to its full potential. Up-regulation of B7-1 (CD80) and B7-2 (CD86) by the dendritic cells in psoriatic lesions suggests a critical role for the CD28/B7 costimulatory system in the pathogenesis of psoriasis. Thus, inhibition of the CD28/B7(CD-80,86) pathway may restrict the inflammatory processes of psoriasis. In this study we used selective antisense oligonucleotides against human CD-80,86 mRNA. Transplanted psoriasis plaques on the SCID mice ($n = 6$) were treated with a cream preparation consisting of 2.5% of each antisense oligonucleotide. Both oligos were 2′-O-methoxyethyl-modified 20 mers with phosphorothioate backbones. The plaques from same patients were transplanted on the control mice ($n = 6$) and treated only with the vehicle of the active cream preparation. Both groups received the topical preparation twice a day for 4 weeks. Significant histological improvement marked by reduction of hyperkeratosis, acanthosis and lymphomononuclear cellular infiltrates was observed in the mice treated with CD80/CD86 antisense oligonucleotide cream. In the mice treated with the active agent the rete pegs changed from 238.56 ± 98.3 μm to 168.4 ± 96.62 μm ($p < 0.05$); whereas in the control group the rete pegs before and after treatment were 279.93 ± 40.56 μm and 294.65 ± 45.64 μm, respectively (p0.1). HLA-DR positive lymphocytic infiltrates and intraepidermal CD8 positive lymphocytes were significantly reduced in the transplanted plaques treated with the CD80/CD86 antisense cream. Here we approached in a unique way to modulate the immune response by targeting the costimulatory molecules. Results of this study indicate the therapeutic potential of topical antisense oligonucleotide treatment in psoriasis and other inflammatory diseases.

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A K17 Peptide Induces Intracellular IFN-γ in Lymphocytes from Psoriasis Vulgaris Patients

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Interferon-γ (IFN-γ) is one of several major regulatory cytokines implicated in lesion formation in patients with psoriasis, an autoimmune skin disorder. Recently, short regions of keratins were reported to induce IFN-γ in circulating T cells in higher numbers from psoriatic patients than from normal volunteers (Gudmundsdottir, *Clin Exp Immunol* 117:580–586, 1999). We are testing similar pieces of synthesized keratin (K) peptides (19 AA each) of K17 and K18 on psoriatic patients' lymphocytes in order to begin quantifying the Type 1 IFN-γ vs. Type 2 Interleukin-4 (IL-4) responses to these keratins. The circulating white blood cells of patients were pulsed for 5 h with either peptide or ionomycin/12-O-tetradecanoyl-phorbol 13-acetate (PMA) or left unstimulated. Induction of IFN-γ, IL-4, and the early activation marker CD69 was then measured by intracellular flow cytometry. These measurements were considered positive when they were greater than unstimulated controls. We found that the K17 peptide induced intracellular IFN-γ and low levels of IL-4 in contrast to an equal concentration of K18 peptide, which failed to induce either cytokine. Despite the differences in cytokine responses, both keratin peptides could stimulate CD69 in T cells, although the K17 response was greater than that of K18. These preliminary experiments provide the basis for designing and evaluating cell-mediated antikeratin responses in psoriatic patients.

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Tissue Distribution and Subcellular Localization of S100A7, S100A11 and S100A10 in Normal and Psoriatic Epidermis

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S100 proteins are a family of small, acidic, calcium-binding proteins that regulate cell differentiation, proliferation, and morphology. Calcium binding alters S100 protein structure and they, in turn, bind to target proteins and regulate signal transduction processes. Eight of these calcium-activated regulatory proteins are expressed in epidermis, and some may be involved in the genesis of proliferation-associated skin disease. S100A7, for example, is highly overexpressed in psoriasis. In addition, selected S100 proteins are known to be components of the keratinocyte cornified envelop (Robinson and Eckert, *JBC* 273:2721, 1998). However, relatively little is known about their subcellular distribution or specific function during differentiation. Our present studies are designed to map the subcellular distribution of S100A10, S100A11 and S100A7 in differentiating normal and psoriatic epidermis. Epidermal sections were paraformaldehyde-fixed and processed for immunohistochemistry or immunoblot analysis. We show that S100A7 and S100A11 localize in the basal and spinous layers in both normal and psoriatic epidermis. Moreover, S100A7 and S100A11 relocate from the cytoplasm, in basal keratinocytes, to the plasma membrane in differentiated spinous layer cells. S100A10 expression is also restricted to the basal and spinous layers; however, it is both membrane-associated and cytoplasmic in these cell layers. Compared to normal tissue, S100A7 expression is markedly elevated in psoriatic epidermis. S100A11 and S100A10 expression, in contrast, are not changed. S100A10, however, it is less abundant than S100A7 or S100A11. These findings indicate that S100 proteins translocate to the plasma membrane during keratinocyte differentiation, suggesting that their ultimate regulatory role is likely to be accomplished at this location.

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Proteasome Inhibitor PS519 Reduces Superantigen-Mediated T-Cell Activation and Severity of Psoriasis in a SCID-hu Model

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Psoriasis is a T-cell mediated skin disease characterized by the infiltration of activated leukocytes and the presence of a distinct inflammatory pattern. Superantigen activation of T-cells plays a pivotal role in the induction of psoriasis. NFκB is a transcription factor which is involved in the induction of many pro-inflammatory molecules known to be up-regulated in psoriasis. We therefore addressed the question whether attenuation of NFκB activation by the proteasome inhibitor PS519 may suppress T-cell mediated responses and may be effective in the treatment of psoriasis. PBMC of 4 controls were stimulated with the superantigen TSST-1 in the presence of nontoxic concentrations of PS519 (0–10 μg per ml). We found a dose-dependent inhibition of lymphocyte proliferation of up to 95% compared to TSST-1 treated cells. Additionally, the expression of T-cell activation markers such as CD69, CD25 and HLA-DR was markedly reduced in PS519 treated, TSST-1 stimulated cultures. The effects of PS519 on the severity of psoriasis was assessed in a xenogenic SCID-hu transplantation model. Lesional psoriatic skin obtained from different donors was transplanted onto SCID mice. After 28 days, SCID mice were treated once daily either with PS519 intraperitoneally (20 μg per mouse; n = 8) or with vehicle for 28 days (n = 4). Thereafter, 20S proteasome activity was determined in the blood and the human transplanted skin examined by immunohistochemistry. As expected, 20S proteasome activity was markedly reduced in PS519 treated mice (0.22 ± 0.01 [pmol AMC per s per mg protein]) compared to untreated mice (1.53 ± 0.05; mean ± SEM). In PS519 treated grafts, normalization of epidermal architecture including loss of papillomatosis and marked reduction of akanthosis was observed. We conclude that the PS519 reduces superantigen mediated T-cell activation *in vitro* and shows antipsoriatic efficacy in the SCID-hu model.

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Human Dermal Migratory CD14+ Cells Differentiate into Langerhans Cells in the Presence of GM-CSF, IL-4 and TGFβ-1

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Langerhans cells (LC) are skin migratory dendritic cells (DC) that, either constitutively or under pathological conditions, traffic to the epidermis where they function as sentinels of the immune system. Although it has been postulated that LC precursors might represent a resident cell population in the skin, there are no previous data confirming this hypothesis. In the present study, we have isolated and characterized a population of human dermal-resident CD14+ cells that actively migrate from human epidermal/dermal explants (mCD14+). The morphological characteristics of different skin migratory cells was determined in cytospins stained with May Grünwald Giemsa and by scanning and transmission electron microscopy. The immune phenotype of CD14+ and CD14– skin migratory cells was analyzed by flow cytometry. The functional characteristics of both cell populations were compared in antigen uptake assays and in the allogeneic mixed leucocyte reactions. Skin migratory CD14+ cells did not adhere to plastic and demonstrated combined morphologic and immunephenotype characteristics of monocytes and myeloid DC markers (expression of CD14, CD68 and CD11b together with S-100, Class-I, Class-II, CD40, CD80 and CD86 molecules, and they lack CD1a, E-cadherin and Birbeck granules (BG)). Functionally mCD14+ cells showed receptor mediated endocytosis and macropinocytosis activities, and they were a very weak stimulators of allogeneic naïve T cells. After short-term culture in GM-CSF + IL-4 + TGFβ-1, mCD14+ cells acquired ultrastructural features (BG and multillamelar compartments) and immunophenotype of epidermal LC (E-cadherin+, CD1a+, and lack of CD14) and they became potent stimulators of allogeneic T cells. Our results show for the first time that skin migratory CD14+ cells constitute a reserve pool of LC precursors in the dermis that are able to differentiate into epidermal LC in the appropriate cytokine environment.

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Cord Blood CD34+ Cells Differentiate into Dermal Dendritic Cells in Co-Culture with Cutaneous Fibroblasts

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Skin is a unique organ that contains two different subsets of dendritic cells (DCs), i.e. Langerhans cells (LCs) and dermal DCs. Our hypothesis is that cutaneous fibroblasts may affect the development of these DCs. We cocultured cord blood CD34+ hematopoietic progenitor cells (HPC) with several human cutaneous fibroblast cell lines (CFCL) without any exogenous cytokines for three weeks. In this culture, HPC increased in number from 8.2 ± 2.1 times, and produced aggregates of cells with dendritic processes (n = 12). They were composed of $56.7 \pm 3.6\%$ HLA-DR + CD14+ CD1a- cells and $12.2 \pm 3.5\%$ HLA-DR + CD1a+ cells, which also expressed CD11b and CD11c. Although some of them were Factor XIIIa+, there were no Lag+ or E-cadherin+ cells. They were potent stimulators in allogeneic T cell activation. There was a significant difference in the ability to induce CD1a+ cells among different CFCL. TNF-α induced the maturation of these DCs with augmented expression of CD86. The subsequent experiment using a dividing chamber suggested a crucial role of direct contact between HPC and CFCL in their growth and differentiation. The study using anti-GM-CSF antibody denied the role of GM-CSF secreted from CFCL in this culture system. Finally, we found that, using the culture system that preferentially stimulated the differentiation of HPC into LC, the coculture with CFCL deviated their differentiation from CD1a + E-cad + Lag + LCs into CD1a + factor XIIIa + E-cad- Lag- dermal DCs. These data suggest that cutaneous fibroblasts support the differentiation of dermal DCs in addition to that of monocytes from HPC.

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Toll-Like Receptor is Differentially Expressed by Murine Langerhans Cells and Splenic Dendritic Cells

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Toll like-receptors (TLRs) are recently discovered surface molecules through which the signaling of bacterial components, such as lipopolysaccharide (LPS) is mediated. Among nine subtypes of TLRs identified so far, TLR2 and TLR4 seem to be of particular importance in LPS signaling. C3H/HeN and C3H/HeJ mice are good and poor responder to LPS, respectively, and C3H/HeJ has the point mutation in TLR4. Langerhans cells (LC) are immature dendritic cells (DC) residing in the epidermis. The expression of TLR2 and TLR4 by murine LC and splenic DC was investigated. The C3H/HeN LC were enriched by panning method using anti $I\text{-}A^k$ antibody (~95% purity assessed by flowcytometry). The splenic DC were obtained through the positive selection by CD11c. The mRNA for TLR2 and TLR4 was detected in both LC and splenic DC assessed by RT-PCR. However, the flowcytometry using the antisera to TLR4 revealed the TLR4 expression was very weak in LC while it was highly positive in splenic DC. CD14 was negative in LC and positive in splenic DC. The production of IL-6 from C3H/HeN DC was highly increased (92.66 ± 10.50 pg per ml vs. 271.12 ± 79.40 pg per ml, $p < 0.05$) by 1 mg per ml LPS. However, that from C3H/HeN LC was not increased (58.96 ± 35.17 vs. 54.17 ± 5.92, not significant) by LPS alone. But, by the combination of 20 mg per ml CD40 ligand, 100 ng per ml IFN-γ and 1 ng per ml TGF-β, the production of IL-6 from C3H/HeN LC was increased (58.96 ± 35.17 vs. 142.71 ± 7.89, $p < 0.05$), and the simultaneous addition of 1 mg per ml LPS to this combination further augmented IL-6 production (142.71 ± 7.89 vs. 246.88 ± 27.54, $p < 0.05$). On the other hand, no significant augmentation was found in C3H/HeJ LC (87.63 ± 22.01 vs. 75.538 ± 22.12, NS) by the additional LPS, indicating this LPS signaling was mediated through TLR4. The similar augmentative and unchanged tendency was found in C3H/HeN and HeJ DC, respectively. Our results verified the expression of TLR4 by murine LC and splenic DC. The difference in the responsiveness to LPS between LC and splenic DC was consistent to the deviated expression of TLR4, together with CD14, between these two DC populations, suggesting the heterogeneity in the TLR expression among the murine DC.

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Increased Expression of Stat4 mRNA and Protein by Activated Murine Skin Dendritic Cells

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Activation of immature dendritic cells (DC), like epidermal Langerhans cells (LC), by microbial components transforms them from antigen acquiring cells into potent accessory cells that can induce primary responses in naïve T cells and shape T cell mediated immunity. mRNA profiling using arrays of known and/or incompletely characterized cDNAs may lead to insight into regulation of DC activation. We purified mRNA from murine fetal skin-derived DC (FSDDC), cells that accurately model LC, before and 24 h after exposure to *E. coli* LPS (100 ng per ml), and relative levels of approximately 9400 transcripts were determined by hybridization to a commercial mouse cDNA array (Incyte Genomics). mRNA encoding Stat4 was one transcript that was dramatically elevated in activated DC. To confirm this, we used real-time quantitative RT-PCR and measured up to 80-fold induction of Stat4 mRNA following LPS stimulation of FSDDC. Western blotting revealed that up-regulation of Stat4 mRNA was accompanied by protein expression. Stat4 mRNA was also detected in LC that matured in culture, as well as in epidermal cells prepared from LPS (50 ng) injected murine ear skin. Concordant reduction of I-A alpha chain and Stat4 mRNAs in suspensions depleted of MCH Class II + cells using magnetic beads indicated that LPS induced Stat4 mRNA expression in LC. Stat4 is essential for IL-12-dependent IFNγ production by T cells, and murine splenic DC have been reported to produce this important T cell regulatory cytokine. However, using identical stimuli and RT-PCR or ELISA, we failed to detect IFNγ mRNA or protein production by murine skin DC (FSDDC and LC). In addition, initial experiments have not revealed surface phenotypic differences between LC from wild type and STAT4 knockout mice, and STAT4 knockout and wild type LC induce allogeneic T cell proliferative responses of comparable magnitudes. Thus, although it is intriguing that Stat4 is induced in activated skin DC, the functional relevance of this observation remains to be determined.

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Study of MHC Class II–Autoantigen Interactions by Confocal Microscopy

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The characterization of the route and location of autoantigen interactions with disease susceptibility class II molecules at the molecular level will help to define the nature of genetic susceptibility and elucidate the early mechanisms of autoimmune induction in diseases such as pemphigus vulgaris (PV). Confocal microscopy allows three-dimensional, real-time imaging of dynamic cell processes. We have transfected the human melanoma cell line Mel JuSo (which express DRA1*0101 constitutively) with the PV associated MHC class II DRB1*0402 that has been coupled to enhanced green fluorescent protein (EGFP). We have used fluorescent techniques to visualize DRB1*0402 intracellularly and at the cell surface in Mel JuSo cells. We have also shown Mel JuSo cells to have active endocytic uptake, utilizing a cyanine 3-transferrin conjugate to visualize the endocytic pathway. Furthermore, we have coupled tetramethylrhodamine to the amino terminus of desmoglein 3 (Dsg3) 190–204 peptide (Dsg3190–204 has been previously reported as a putative autoantigenic epitope in PV). We are coculturing DRB1*0402-EGFP expressing Mel JuSo cells with the tetramethylrhodamine-Dsg3190–204 conjugate to track the intracellular interactions of class II molecules with autoantigens by 2 color confocal analysis. The intracellular location of MHC class II–peptide interaction is being studied by three color analysis using a panel of cyanine 5-conjugated antibodies directed against markers for specific subcellular compartments. Confocal microscopy offers an innovative platform to systematically study the interaction of a specific autoantigen with known class II susceptibility molecules at the intracellular level. By investigating the intracellular locations and biochemical parameters required for autoantigen interaction with disease susceptibility class II molecules, we seek to strengthen our understanding of MHC-peptide–T cell interactions in autoimmunity and further our goal of developing viable immunotherapeutic strategies.

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Expression and Function of Adenosine-Receptors in Human Dendritic Cells

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Dendritic cells (DCs) are specialized antigen presenting cells characterized by their ability to migrate into target sites, process antigens and activate naive T cells. Here, the biological activity and intracellular signaling of adenosine was analyzed. Using RT-PCR analyses, mRNA-expression of the adenosine-receptors A1, A2a and A3 in immature human DCs was revealed. Functional experiments after adenosine-stimulation showed chemotaxis, intracellular calcium transients and actin polymerization, but no activation of adenylyl-cyclase in immature DCs. Studies with selective receptor agonists indicated that chemotaxis, calcium transients and actin polymerization were mediated via A1- and A3-receptors. Maturation of the DCs induced by LPS resulted in down-regulation of the A1- and A3- receptor mRNA, while the A2a-receptor mRNA was still expressed. Yet, in LPS-differentiated DCs adenosine and the A2a-receptor agonist stimulated adenylyl-cyclase activity, enhanced intracellular cAMP levels and inhibited IL-12 production. In contrast, adenosine and the selective A1- and A3-agonist failed to induce chemotaxis, actin polymerization and calcium transients in LPS-differentiated DCs. This findings indicate that adenosine has important biological effects in DCs activity as chemotaxin towards immature DCs and modulating IL-12 production in mature DCs.

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Particulate Antigen Delivery Reveals Functional Differences Between *In Vivo* Generated Myeloid and Lymphoid Dendritic Cells

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CD8+ lymphoid (LDC) and CD8-myeloid (MDC) splenic DC populations are hypothesized to have distinct roles in eliciting tolerogenic and stimulatory effects, respectively, on the immune response, although direct *in vitro* or *in vivo* functional evidence for these effects has not been clearly established. In this study, we systematically compared the uptake, processing, and presentation of particulate antigens between LDC and MDC *in vitro* and *in vivo*. Significant differences between LDCs and MDCs are observed both *in vitro* and *in vivo* when examining phagocytosis of particulate latex microsphere antigens using a quantitative cytofluorometric approach. While LDCs were found to be deficient in the capacity to capture particulates irrespective of their maturation status, we were surprised to discover that both phenotypically immature and mature MDCs could internalize particulate antigens in an inhibitable fashion. Class I-restricted OT-I T cell proliferation and class II-restricted DO.11 cell stimulation assays demonstrated that intracellular proteolytic processing and presentation of antigen-loaded particles is found only in MDCs. Finally, we demonstrated that difference in particulate antigen uptake and presentation of LDCs and MDCs was retained in unperturbed splenic DC populations *in vivo*. Our data suggest that the specific role of bystander LDCs in immunologic priming or tolerance induction remains in question, while MDCs may directly participate the initiation of antigen-specific immunity by internalizing and processing blood-borne antigens, specifically particulates, *in vivo*.

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The Langerhans/Dendritic Cell-Associated Protein, 3C5, Structurally Resembles Subunit d of the Vacuolar-ATPase Complex, but is Functionally Distinct

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For efficient presentation to T cells, protein antigens (Ag) have to be processed by Ag presenting cells (APC) within acidic vacuolar compartments fueled by the proton pump activity of vacuolar (V)-ATPase complexes that are, in turn, made up of multiple subunits including subunit d. By subtractive cloning, we recently identified a novel protein (3C5) expressed selectively by mouse Langerhans cells (LC) and dendritic cells (DC). 3C5 shares 67% sequence homology with mouse subunit d (msd). In addition, 3C5 transfected into COS cells augments the activity of V-ATPase complexes. To better characterize 3C5 function, we took advantage of a yeast strain (Vma6Δ) that lacks the VMA6 gene, rendering Vma6Δ unable to grow in media with neutral pH or with glycerol. We asked whether 3C5 or msd can substitute functionally for the VMA6 gene, allowing Vma6Δ cells to grow in media of neutral pH or containing glycerol. msd-transformed Vma6Δ cells grew as vigorously as the wild type, whereas 3C5-transformed Vma6Δ cells failed to grow (just like Vma6Δ alone). To account for this failure, we measured 3C5 protein expression and assembly of V-ATPase complexes in these transformed cells. 3C5-transformants exhibited high levels of C35 protein. However, they produced very low levels of assembled V-ATPase complex proteins, whereas msd-transformants and wild type cells displayed high levels. Thus, although 3C5 resembles msd structurally, it is functionally distinct. Finally, we hypothesize that 3C5 in LC and DC is a regulator of V-ATPase complex activity (rather than a subunit of the V-ATPase complex) in these cells, thus contributing to their potent APC capacity.

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Histamine Receptor on Human Dendritic Cells: Identification and Coupling to Chemotaxis and Cytokine Release

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Histamine, is a well-known inflammotry mediator, which has been implicated in varoius immunoregulatory effects that are poorly understood. Thus, we investigated the biological activity and intracellular signaling of the histamine on Dendritic cells (DCs), which are specialized antigen presenting cells characterized by their ability to migrate into target sites, process antigens and activate naive T cells. Here we could show by using RT-PCR that immature and matured human DCs express the spezific mRNA of the H1-, H2- and H3- histamine-receptors. Functional experiments after histamine-stimulation showed chemotaxis, intracellular calcium transients and actin polymerization, but no activation of adenylyl-cyclase and cytocine production in immature DCs. Studies with receptor selective agonists indicated that activation of all 3 receptor subtypes induced calcium transients. In contrast actin polymerization and chemotaxis were mediated via H1- and H3-receptors, while the activation of the H2-receptor with Dimaprit even resultes in a inhibition of migration of immatured DCs. Maturation of the DCs induced by LPS resultes in the loss of histamine induced cell responses chemotaxis, actin polymerization and calcium transients. Whereas now histamine dose-dependently enhanced intracellular cAMP levels and IL-10-production while it inhibited IL-12 and TNFα secretion. Stimulation with the different receptor-agonists showed, that the modulation of IL-12 and IL-10 production by histmaine were mediated through the H2- and H3-receptor. In contrast all three receptor subtypes seemed to be invovled in the inhibition of TNFα production. This findings indicate that histamine has important biological effects in DCs activity as chemotaxin towards immature DCs and modulating IL-10, IL-12 and TNFα production in mature DCs.

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A Mouse Model of Allergic Asthma Induced by Adoptive Transfer of Antigen-Pulsed Dendritic Cells

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Though allergen-specific Th2 cells play a central role in the pathogenesis of allergic asthma, the mechanism leading to Th2 cell priming remains unknown. While dendritic cells (DC) are the most potent type of antigen presenting cells capable of inducing Th1 immune responses, it is not clear whether they play a role in the generation of Th2 responses. To determine whether DC can induce dominant Th2 cell responses, we chose to study the role of DC in the initiation of mouse allergic asthma. We injected naive C57Bl/6 syngeneic mice ip. with OVA-pulsed wild type DC, MHC class II–/– DC or irradiated RMA tumor cells on day 0, followed by aerosolized OVA 7 and 14 days later. The DC-enriched spleen cell populations consisted of 60–90% CD11c+ cells with high expression of costimulatory and adhesion molecules. Only 5% of this population was CD8a+ suggesting cells of predominantly myeloid origin. Functional analysis of these DC *in vitro* revealed that purified OVA-pulsed wild type DC stimulated OVA-specific CD4+ BO-97.11 T hybridoma cells to produce IL-2 compared with unpulsed DC. Upon adoptive transfer of OVA-pulsed wild type DC, we observed lung eosinophilia (60%) 48 h after the last aerosol challenge. In addition, OVA-specific serum IgE and IgG1 levels were elevated in these mice. No response was induced by unpulsed cells or OVA-pulsed RMA tumor cells. Sensitizing with OVA-pulsed MHC class II–/– DC led to increased numbers of lung infiltrating lymphocytes similar to wild type DC but without induction of eosinophilia and lower levels of IgE and IgG1. These results suggest that dendritic cells are involved in the induction of Th2-mediated immune responses and may be an interesting target for therapeutic strategies aiming at interfering with atopic tissue inflammation.

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Restraint-Induced Modification of Langerhans Cell Morphology and Migration in BALB/c Mice is Dependent Upon Timing of Restraint

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The timing of a stressful event with respect to antigen exposure affects the development of the immune response. We have reported that restraint applied to a naive mouse prior to sensitization (day 1) decreased chemical-induced changes in ear swelling and lymphocytosis and restraint applied during sensitization (days 1 and 2) or prior to challenge (day 6), increased ear swelling. We hypothesized that these dichotomous effects of restraint would be reflected in changes in Langerhans cell (LC) migration and morphology. To compare the effects of restraint on LC in naive and DNFB-treated mice, we sensitized mice onto the back with 0.5% DNFB on days 1 and 2 and challenged onto the ears with 0.25% DNFB on day 6. Mice were restrained for 2 h immediately prior to chemical application on day 1 or on day 6 prior to challenge. LC morphology and migration were examined in epidermal sheets by counting the number of FITC-conjugated Ia stained cells per mm^2 at 24 h after DNFB-challenge. To assess the effect of restraint on T cell proliferation *in vivo*, 24 h following challenge, T lymphocyte proliferation was examined using the local lymph node assay. We determined that DNFB induced a significant reduction in the number of epidermal LC at 24 h. Restraint on day 1 resulted in the retention of LC in the epidermis and the LC had longer dendritic processes than nonrestrained mice. However, LC from mice restrained on day 6 demonstrated a dendritic cell like-morphology similar to nonrestrained mice but showed a significant reduction in epidermal LC numbers 24 h after DNFB challenge. Furthermore, we demonstrated a significant two-fold decrease in T cell proliferation in mice restrained on day 1 and an elevation of T cell proliferation in mice restrained on day 6. These data suggest that acute restraint stress applied prior to sensitization modulates epidermal LC morphology and migration and T cell proliferation differently than restraint applied prior to challenge.

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Intact Lymphoid Structure and Germinal Center Formation are Not Crucial for Mounting an Antigen Specific Humoral Response in CD18 Null Mice

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β-2 integrins play a central role in host defence and if specifically mutated lead to a disorder known as leukocyte adhesion deficiency 1 (LAD1) in humans. Mice with a CD18 null mutation suffer from recurrent bacterial infections, impaired wound healing and skin ulcers closely resembling human LAD1. We here report on the impact of CD18 deficiency on the organization of lymphoid tissue, B cell differentiation, germinal center formation and T-dependent B cell response. CD18 deficient and wildtype (wt) control mice were immunized with the T-dependent antigen nitrophenylacetate (NP) chicken gammaglobulin to analyze NP hapten-specific antibody production. All animals were re-injected with the soluble antigen at day 34 to further study the secondary immune response. The detection of serum NP-specific antibodies showed only a slightly retarded and decreased primary response in in CD18 null mice, whereas class switch and affinity maturation occurred as in wt mice. In the secondary immune response virtually no difference to wt controls existed indicating a normal memory B cell generation and function. These results were completely unexpected, as conventional histology and immunohistochemistry with stainings for proliferation (Ki-67), germinal center B cells (peanut agglutinin), T-cell and dendritic cell markers of all major lymphoid tissues which were performed at three time points after primary immunization had revealed a completely resolved architecture of the lymphoid tissues without any initiation of classical germinal center reactions. In ultrastructural analysis the tight membrane adhesions (< 5 nm) between lymphocytes and dendritic cells observed in wt mice (n=6) were missing in CD18 null mutants (n=6) in all lymphoid tissue sections analyzed (lymph nodes, spleen, Peyer's patches, tonsils). However, rudimentary bridges of membrane contacts existed. We here provide evidence that formerly described lymphoid structures and germinal center formation are not necessarily required to mount an antigen specific immune response in CD18 null mice, but that residual structures can compensate. (BMBF 01 KS 9502)

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Rho GTPases are Key Regulators of Dendritic Cell Morphology and Function

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Dendritic cells (DCs) are characterized by their irregular, dendrite (i.e. lamellipodium and filopodium)-bearing plasma membrane and by their unique ability to activate naïve T lymphocytes. The latter function is linked to their high migratory capacity and their abundant display of MHC and costimulatory molecules. Interestingly, cell shape, migration, and export of MHC products to the cell surface of DCs require actin assembly. Molecules that mediate actin polymerization, and, thus, may control DC phenotype and function are small Rho GTPases. To study the importance of individual GTPases in DCs, stem cell-derived Langerhans cell-type DCs were transfected with constructs encoding myc-tagged dominant negative mutants of Cdc42 (N17-Cdc42), Rac1 (N17-Rac1) or RhoA (N14-RhoA). CD40 ligand-stimulated DC transfectants showed stable expression of the myc-tagged transgenes over several days and survived at similar rates as empty vector transfected control cells. By confocal microscopy, we observed dramatic alterations of the cellular cytoskeleton imposed by the various constructs. N17-Cdc42+ DCs were essentially round without filopodia or lamellipodia and displayed an only poorly developed actin cytoskeleton. N17-Rac1+ and N14-RhoA+ DCs displayed collapsed lamellipodia and thinned surface-associated actin, respectively. In contrast, constitutively active GTPase mutants induced lamellipodia formation (L61-Rac1), short membrane protrusions (L63-RhoA) and, most strikingly, long, actin-bearing dendrites/filopodia (L61-Cdc42) in monocytes. Thus, by phenotype, these monocytes were hardly discernable from DCs. Furthermore, DCs transfected with dominant negative mutants of Rho-GTPases showed reduced migration to chemokine stimuli and were also impaired in their ability to stimulate T cells. Surprisingly, DCs require functional Cdc42 for the stimulation of CD8+, but less so, for CD4+ T cells. Apparently, this effect is mediated by reduced MHC class I surface export of this molecule from (post)Golgi compartments but is not due to destabilization of surface MHC class I. In contrast, MHC class II surface expression and IL-12 secretion were hardly affected. In summary, DC morphology and DC function are co-ordinately controlled by similar, if not identical, signaling modules, i.e. Rho GTPases. Moreover, Cdc42 seems to be of particular importance for DC-T cell communication and, thus, for T-cell-dependent immunity.

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IL-10-Treated Human Dendritic Cells Induce Anergic CD4+ and CD8+ T Cells with Antigen-Specific Suppressor Activity

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Recently, we demonstrated that IL-10-treated dendritic cells (DC) induce an alloantigen- or peptide-specific anergy in various CD4+ and CD8+ T cell populations. In this study we wanted to analyse whether these anergic T cells have immunoregulatory functions. Coculture experiments revealed that alloantigen-specific anergic CD4/8+ T cells suppress the proliferation of naive syngeneic T cells in a dose dependent fashion. The same effect was observed if tyrosinase-specific cytotoxic CD8+ T cells or the haemagglutinin-specific CD4+ T cell clone HA1.7 were cocultured with anergic T cells of the same specificity. In contrast, anergic T cells did not mediate an antigen-independent bystander inhibition. Functional analysis revealed that previously activated tyrosinase-specific CD8+ T cells cocultured with anergic tyrosinase-specific CD8+ T cells failed to lyse a tyrosinase-expressing melanoma cell line. Suppression was dependent on cell to cell contact between anergic and responder T cells and required activation by antigen-loaded DC. Supernatants of anergic T cells did not mediate T cell suppression. Inhibition could be overcome by addition of IL-2 or anti-CD3-/CD28-mAb and was blocked by CTLA-4-Ig or anti-CD86-mAb. Furthermore, anergic T cells displaying increased expression of CTLA-4 surface molecules were involved in the suppressor activity. Taken together, our experiments demonstrate that anergic T cells induced by coculture with IL-10-treated DC mediate antigen-specific suppression of T cells. Induction of anergic T cells might be exploited therapeutically for suppression of cellular immune responses in allergic or autoimmune diseases with identified (auto-) antigen.

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Adhesion of $CD34^+$-Derived Dendritic Cells to Human Dermal Microvascular Endothelial Cells is Down-Regulated upon Maturation and Depends on CD11a, CD11b, CD36

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Dendritic cells (DC) function as sentinels of the immune system. When bone marrow-derived DC precursors reach their target tissue, they need to bind and migrate out of *microvascular* endothelial cells, in particular. To determine the adhesive properties of DC at various differentiation stages to human dermal microvascular endothelial cells (HDMEC), DC were generated from human cord blood-derived $CD34^+$ hematopoietic progenitors by culture in the presence of GM-CSF, TNF-α and hSCF. To enhance DC maturation, cells were additionally exposed to a defined cocktail of IL-1β (2 ng per ml)/IL-6 (1000 U per ml)/TNF-α (10 ng per ml)/PGE2 (1 μg per ml) ("cocktail DC"). Adhesion was assessed and quantified by phase contrast microscopy and by means of a fluorimetric adhesion assay. Significantly more immature (37.8 ± 9.7%) than mature DC (13.8 ± 6.3% for conventionally matured DC and 2.5 ± 0.87% for "cocktail DC") bound to unstimulated HDMEC. HDMEC pretreatment with TNF-α and IFN-γ resulted in an enhanced attachment of both immature and mature DC by 50–160%. Flow cytometric analysis revealed that mature DC are characterized by the lack of CD31, CD36, CD45RA and CLA expression and by the low expression of CD11a, CD11b and CD49d. Blocking mAb's against CD11a, CD11b, and CD36 markedly inhibited DC binding (mean inhibition 65–70%), whereas anti-CD49d Ab's did not. Simultaneous application of these mAb's did not potentiate inhibition. Our data support the hypothesis of immunosurveillance with selective recruitment of immature blood DC at sites of cutaneous inflammation. They might have potential relevance in the development of immunotherapy strategies for cancer indicating the inefficacy of intravenous DC application.

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Differential Effects of LPS and TGF-β on the Production of IL-12 p40 and IL-6 of Mouse Langerhans Cells, Spleen-Derived Dendritic Cells and Macrophages

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The response of Langerhans cells (LC)/Dendritic cells (DC) to bacterial lipopolysaccaride (LPS) is the best example of innate recognition. However, contradictory results have been reported on the ability of LC/DC to respond to LPS. Here, we examined modulatory effects of LPS on IL-12 p40 and IL-6 production of highly purified LC (95%), spleen-derived $CD11c^+$ DC and peritoneal exudate macrophages (Mφ) by ELISA. Low dose LPS (1 ng per ml) up-regulated IL-12 p40 production of anti-CD40/IFN-γ-stimulated Mφ, and it also up-regulated IL-6 production of anti-CD40/IFN-γ-stimulated DC and Mφ. In contrast, low dose LPS up-regulated neither IL-12 p40 nor IL-6 production of stimulated LC. Since LC did not express CD14 and spleen-derived $CD11c^+$ DC expressed low density of CD14, it is likely that LC, DC and Mφ respond differently to low dose LPS depending on the density of cell surface CD14 expression, which is at least known to be involved in LPS-induced Mφ activation. However, high dose LPS (1 μg per ml) up-regulated IL-6 production of LC, suggesting the ability of LC to respond to LPS. We also explored the immunomodulatory effects of transforming growth factor (TGF)-β on stimulated LC and Mφ, since TGF-β is known to inhibit signalling of LPS-induced inflammatory responses, especially in Mφ. As has been expected, TGF-β_1 down-regulated IL-12 p40 and IL-6 production of anti-CD40/IFN-γ/LPS-stimulated Mφ. TGF-β_1 up-regulated IL-12 p40 production of anti-CD40/IFN-γ-stimulated Mφ, but did not modify IL-6 production of anti-CD40/IFN-γ-stimulated Mφ. In contrast to Mφ, TGF-β_1 up-regulated IL-6 production of anti-CD40/IFN-γ/LPS (1 μg per ml)-stimulated LC, and also up-regulated IL-6 and IL-12 p40 production of anti-CD40/IFN-γ-stimulated LC. These results suggest that TGF-β is the key cytokine in the skin innate immunity by up-regulating LC secretion of IL-12 and IL-6, which are essential for activating cell-mediated and humoral immunity.

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The Negative Effects on Human Dendritic Cells: Roles of Endothelial Interleukin-8

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Since high levels of interleukin-8 (IL-8) had been detected both in serum and *in situ* of melanoma patients in our study,it was of interest to further explore its impacts on dendritic cells (DC),which are the most important antigen presenting cells in the body. Human endothelial cells and monocytes can produce and secrete two forms of IL-8, respectively, with different compositions and distinct biological characteristics by stimulation of TNF-|Á. These two types of IL-8 of different origins were added to the routine DC cultures at an early stage (Day 3) or at a late stage (Day 7) maintaining till supernants were collected on day 9. Here we reported for the first time the negative effects of endothelial IL-8 on human DCs. Endothelial IL-8, which if added at an early stage to the DC cultures, could inhibit DC maturation with significantly decreased expressions of CD40, CD80, CD83 and HLA-DR; while CD14, a marker for macrophage, expression was obviously up-regulated with corresponding morphologic changes. Such effects were also demonstrated by greatly reduced stimulating indexes of allogeneic mixed lymphocyte reactions and interleukin-12 secretions by DCs. Moreover,they could be selectively blockaded by anti-IL-8 monoclonal antibodies. But monocytic IL-8 did not show any inhibitory impacts on DCs. In conclusion, DCs couldn't undergo terminal maturation in response to endothelial IL-8 that had five extra N-terminal amino acids lacking in monocyte-derived IL-8. This was a novel function of endothelial cells involving melanoma formation and development of tumors by escape from immune surveillance.

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Fractalkine Induces Chemotaxis and Actin Polymerization in Human Dendritic Cells

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Dendritic cells (DCs) are considered as principle initiators of immune responses. They are able to migrate into target sites, process antigens and activate naive T cells. Here, the chemotactic activity and intracellular signaling of fractalkine was analyzed and compared to well known chemotaxins. The mRNA-expression of G protein-coupled CX3CR-1 was analyzed by RT-PCR. Chemotaxis was measured in 48 well Boyden chambers and actin polymerization by flow cytometry. The mRNA-expression of CX3CR-1 in immature and mature DCs has been analyzed. Fractalkine elicited actin polymerization and chemotaxis in a dose-dependent manner in DCs independent from their state of maturation. These results show that immature and mature DCs express mRNA for the CX3CR-1 and that fractalkine induces chemotaxis and migration associated actin polymerization in immature as well as in mature DCs, contrasting the action of other chemokines such as RANTES or MIP3-β which act only on distinct maturation states of DCs. These findings suggest a role of this novel chemokine and CX3CR-1 in the recruitment of DCs.

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Functional Leptin Receptor is Expressed on Human Blood-Derived Dendritic Cells (BDC) and Leptin Promotes Maturation of BDC

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Leptin is a 16-kDa peptide hormone synthesized mainly in adipose tissue to regulate body weight. Although leptin receptor (ObR) is widely distributed including hematopoietic cells, its functional isoform (ObRb) is restricted to certain cells such as T cells. Leptin enhances alloproliferative response by T-cells, especially of its memory subset, and biases T-cell response towards a Th1 type. In this study, we investigated the expression of ObRb on BDC and the effect of leptin on their maturation. BDC was generated from CD14-selected human peripheral blood mononuclear cells (PBMC) by culturing with GM-CSF (100 ng per ml) and IL-4 (100 ng per ml) for 6 days. Expression of ObRb mRNA as well as ObR mRNA was detected by RT-PCR with appropriate primers. Maturation was determined by measuring the levels of cell surface molecules on BDC by flow cytometry after 18 h culture with 0, 3, 10, 30, or 100 nM of leptin in the culture medium, and by the expression of mRNA for proinflammatory cytokines after 4 h-stimulation by leptin. ObRb mRNA could be detected on BDC but the expression level was lower than that of PBMC. In contrast, ObR mRNA level was comparable between BDC and PBMC. High doses of leptin up-regulated the expression of CD54, CD83 and CD86 on BDC and leptin enhanced their expression of IL-1beta, IL-6 and IL-8 mRNA in a dose-dependent manner. These results suggest a possible role played by leptin in the regulation of cutaneous immune response by stimulating dendritic cell maturation *in vivo*.

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R848 Activates Immature Dendritic Cells *In Vitro* and Modulates the Sensitization and Effector Phase of Murine Contact Hypersensitivity (CHS) Responses

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R848 is a 100× stronger derivative of the topical immunomodulator Imiquimod, the active substance of ALDARA(tm). To further explore its mechanism of action, we tested the effects of R848 on the development of antigen presenting dendritic cells (DC) *in vitro* and in different CHS models *in vivo*. Non-adherent bone marrow cells from Balb/c mice were cultured with GM-CSF/IL-4+/− graded doses of R848 for 6 days (development). On day 6 floating cells were harvested, assayed for phenotype/function and replated for additional 24–48 h in GM-CSF/IL-4+/− R848 (maturation). During the development of DC from bone marrow precursors, R848 was an inhibitor of DC proliferation and strongly reduced cell yield after 6 d of culture. Nevertheless, cells recovered from the cultures were almost indistinguishable from untreated controls by surface molecule expression, in their ability to stimulate proliferation of allogeneic T cells in mixed lymphocyte responses (MLR), or in their endocytotic capacity, but produced much larger amounts of TNFα, IL-6 and IL-12. DC which had been incubated with R848 during the maturation phase only were more effective inducers of T cell proliferation as compared to untreated DC or cells permanently in contact with R848. In both cases, R848 incubation drastically increased the capacity of DC to stimulate IFNγ production by allogeneic T cells in MLR. *In vivo*, R848 was given i.p. at the time of sensitization or elicitation of CHS, and ear swelling responses were measured 24 h later. R848 strongly enhanced sensitization, but intriguingly, the elicitation phase of CHS to the allergen FITC was potently inhibited. Thus, while R848 disturbs the development of DC from their precursors, it strongly enhances their capacity to induce Th1 responses *in vitro* and differentially modulates the induction and elicitation phases of murine CHS.

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Internalization and Presentation of Particulate Antigens by Activated Bone Marrow-Derived Dendritic Cells

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Antigen delivery to mature myeloid DC populations has received little attention but may constitute an effective strategy for inducing antigen-specific immunity. We exposed BMDCs differentiated in GM-CSF/IL-4 to potent maturation signals including activating CD40-IgM to examine the capacity of dendritic cell populations to functionally internalize, process, and present distinct soluble and particulate antigen formulations. Our findings support conclusions from previous studies that soluble ligand uptake and proteolysis is greatly diminished in activated DCs. However, when examining for phagocytosis of 1 μm particulates using a sensitive, quantitative cytofluorometric assay, we find that particle uptake is retained within both immature, and enriched populations of mature BMDC. Approximately 12–33% of activated BMDCs are associated with the phagocytic activity observed. Confocal microscopy of mature DC fluorescently stained to identify lysosomes demonstrates that particulate internalization by DCs results in cytoskeletal remodeling and lysosome cluster disruption in a time-dependent manner. Live dendritic cell filming of particle phagocytosis by DC labeled with Lysotracker reagent supports these findings. Importantly, we observe that activated, mature DCs process and presented particulate OVA through both MHC class I and class II restricted pathways to antigen-specific T-cell hybridomas. Taken together, these data argue strongly that phagocytosis is a constitutive and tightly regulated process in both immature and now mature BMDCs, but that ultimate presentation of internalized particulate antigens in activated myeloid DCs is influenced by local microenvironmental cues.

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Cross Priming is an Important Mechanism for T-cell Priming in Cutaneous Genetic Immunization

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To evaluate the role of cross-priming in immune induction following gene gun immunization of mouse skin, the keratin K14 promoter was used to drive expression of a model antigen, cytosolic chicken egg ovalbumin (OVA489), specifically in keratinocytes. The K14 promoter has been previously characterized for its specificity for stratified basal layer keratinocytes. Here we use RT-PCR to show that this promoter does not drive expression in skin dendritic cells (DCs), verifying that antigen-encoding genes driven by this promoter are appropriate tools to study the contribution of cross-priming to immunity in our cutaneous vaccination model. We make use of an *in vivo* assay for antigen-specific proliferation, using adoptive transfer of CFSE fluorescent dye-labeled OVA-specific OT-1 CD8+ T-cells from TCR transgenic mice into naïve syngeneic recipients. Sequential halving of CFSE fluorescence *in vivo* upon cell division can be used to assess the CD8+ proliferative response generated in immunized animals through cross-priming. To our knowledge, we are the first to describe the use of this system for a functional quantitative comparison of T-cell proliferation between treatment groups. We demonstrate that cross-priming through genetic immunization results in a robust proliferative response of antigen-specific OT-1 T-cells. In agreement with these results, standard CTL assays demonstrated that immunization with pcK14-OVA489, encoding cytoplasmic OVA driven by the K14 promoter, resulted in antigen-specific lysis of syngeneic target cells. Time-course experiments suggest that cross-priming requires more time for immune induction, which may be attributable to the time required for repopulation of skin DCs following the initial wave of migration. These results demonstrate that DCs in skin can efficiently take up antigen from transfected keratinocytes for presentation through the MHC-I pathway, resulting in CD8+ effector T-cell proliferative and cytolytic responses. Collectively, these data imply that while direct transfection of dendritic cells may be an important goal for the development of immunotherapeutic strategies, cross-priming is a major mechanism for T-cell priming after biolistic cutaneous genetic immunization. They suggest that the immunogenicity of foreign proteins expressed in keratinocytes may be problematic for the ongoing development of gene therapy strategies.

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Langerhans Cells are Susceptible to Fas-Mediated and Antigen-Specific T Cell-Mediated Apoptosis

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We examined the response of Langerhans cells (LC) to stimuli of apoptosis pathways of activation induced cell death (AICD) and passive cell death (PCD). Fas cross-linking with Jo2 mAb or antigen–specific interaction with a CD4+ T cell hybridoma, 3A9 were used as AICD stimuli, while staurosporine was used as a PCD stimulus. The murine LC line XS106 and LC obtained from A/J mouse skin organ cultures were examined. Flow cytometric analysis revealed that both XS106 cells and LC expressed Fas, which was further inducible by LPS. Fas expression levels correlated with susceptibility to apoptosis by crosslinking with Jo2 mAb, resulting in 80% cell death of LC and LPS-treated XS106 in 20-h cultures, as indicated by Annexin V and 7AAD staining. When XS106 cells were cultured for 20 h in the presence of the antigen hen egg lysozyme (HEL), or with the HEL-specific CD4+ T cell hybridoma, XS106 cell death was not detected. However, when both HEL and 3A9 T cells were cocultured, 50% of the XS106 cells were apoptotic. Similarly, 65% of LC underwent apoptosis after only 12 h of antigen–specific interaction with 3A9 T cells. Overexpression of the human Bcl-xl gene by adenovirus infection of XS106 cells did not confer resistance to T cell-mediated apoptosis, but staurosporine-induced apoptosis was effectively inhibited. These results support a role for immune-mediated apoptosis in regulating LC function.

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Subcutaneously (SC)-Injected, CXCR5-Transduced Bone Marrow-Derived Dendritic Cells (BMDC) Traffic to B Cells Zones of Lymph Nodes (LN) and Modify Antigen-Specific Immune Responses

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Specific chemokine expression in the B and T cell zones (TCZ) of the LN contribute to the development of these distinct anatomical regions. The formation of B cell zones (BCZ), for example, depends on B lymphocyte chemoattractant (BLC) expression in the BCZ of LN and the expression of CXCR5 by B cells. Our previous data showed that skin-derived migratory DC express CXCR5, respond to BLC *in vitro*, and migrate to BCZ in addition to TCZ in LN *in vivo*. Murine BMDC, by contrast, express 10-fold less CXCR5, do not migrate in response to BLC, and migrate exclusively to TCZ in LN *in vivo*. To address whether functional expression of CXCR5 by BMDC alters their trafficking dynamics and influences immune responses, we transduced BMDC with CXCR5 and GFP using a bicistronic retroviral vector (transduction efficiency ~60%). CXCR5-BMDC were responsive to BLC *in vitro* in chemotaxis assays (3-fold over nontransduced BMDC, $p < 0.01$). When injected into the footpads of mice, CXCR5-BMDC migrated not only to TCZ, but also to BCZ, in the draining LN as detected by confocal microscopy. CXCR5-BMDC or BMDC transduced with vector alone were pulsed with keyhole limpet hemocyanin (KLH) and injected SC into Balb/C mice to induce specific cellular and humoral immune responses manifested by footpad swelling in response to KLH injection and KLH-specific Ig production, respectively. In response to KLH challenge, mice injected with CXCR5-BMDC ($n = 5$) demonstrated 23% less footpad swelling ($p < 0.05$). CXCR5-BMDC produced 50% more KLH-specific IgG ($p < 0.05$) and 143% more KLH-specific IgM ($p < 0.01$) than vector-transduced BMDC ($n = 5$). Thus, expression of CXCR5 alone is sufficient to direct BMDC to BCZ of LN *in vivo* and modifies antigen-specific immune responses induced by BMDC vaccination. Therefore, expression of CXCR5 in skin migratory DC *in vivo* may influence immune responses generated against cutaneous antigens.

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UV-B Irradiated Dendritic Cells Cannot Tolerize Hapten-Specific or TCR-Transgenic Murine CD8+ T Cells

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The recognition of CD8+ T cells as the main effector cells in contact allergy to a number of chemicals and drugs necessitates the development of strategies for tolerance induction in CD8+ Tc1 cells. UV-B irradiation of the skin and *ex vivo* Langerhans cells before antigen-application was shown to tolerize mice for contact allergens. We have used UV-B irradiated bone marrow derived dendritic cells (UV-B DC) to induce tolerance in CD8+ T cells specifc for the contact allergen TNP and others in the mouse. In contrast to the successful tolerization of naive CD4+ Th1 cells in some transgene systems, naive CD8+ T cells as well as established Tc1 effector cells could not be tolerized by TNP-modified UV-B DC *in vitro*. In addition, i.d. injection of TNP-modified UV-B DC in a contact hypersensitivity model *in vivo* also failed to induce allergen-specific tolerance in CD8 T cells. To confirm this is a general phenomenon for murine CD8+ T cells we made use of two transgenic TCR-systems: H-2Db/LCMV GP p33-specific P14 mice and additionally the H-2Kb/Ova-specific OT-1 mice. *In vitro* priming experiments showed a reduction of priming capacity by a factor of 10–100 by UV-B DC, but no tolerance induction in rechallenge experiments. UV-DC also failed to tolerize an established CD8+ TC clone. To preliminary results *in vivo*, UV-B DC were not successful in tolerising naive p33-specific CD8+ TC in an adoptive transfer system. Although immunization of the adoptive host with UV-B DC elicited no significant primary proliferative response of the transgenic donor T cells, these T cells proliferated upon rechallenge after 5 weeks. Taken together, bone marrow derived UV-B DC do not induce tolerance in CD8 T cells at least in the investigated systems. We have to think about alternative strategies. This work was supported by the Klinische Forschergruppe "Pathomechanismen der allergischen Entzündung" BMBF FKZ: 01GC9701/7.

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Up-Regulation of Ia^+B220^- Lymph Node Cell Costimulatory Molecules Induced by UVB is Not Further Enhanced by DNFB

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Low-dose *in vivo* UVB exposure results in the suppression of contact hypersensitivity (CHS) and tolerance to an antigen sensitized through the UV-irradiated site. Because costimulatory molecules on antigen-presenting cells (APC) and IL-12 play important roles in inducing Th_1 cells, we analyzed CD40, CD80 and CD86 on APC by flow cytometry and IL-12 p40 by RT-PCR in lymph nodes (LN) where sensitization occurs. C3H/HeN mice were painted with 0.5% DNFB, as a sensitizing agent, on back skin without UV irradiation or 48 h after a tolerogenic UVB irradiation (72 mJ per cm^2). Draining LN were prepared for three-color flow cytometry and RNA extraction. Since Ia^+ cells include a large number of B cells and B cells express CD40, CD80 and CD86, we used B220 (B cell marker) to select a B220 negative population ($B220^-$). The percentage of Ia^+B220^- cells was slightly up-regulated 18 h after sensitization (0.35 ± 0.05 vs. 0.44 ± 0.07). The percentage of Ia^+B220^- cells 66 h after UV irradiation in nonsensitized mice was significantly up-regulated (0.35 ± 0.05 vs. 0.48 ± 0.03, $n = 4$, $p < 0.05$). At 66 h post-UV, IL-12 p40 mRNA was also detected by RT-PCR. However, sensitization on UV skin did not result in a significantly increased percentage of Ia^+B220^- cells (0.46 ± 0.08) compared to either sensitized or UV treated alone. Increased expression of costimulatory molecules was also observed upon sensitization and UV treatment. No additive increase in CD40 expression on Ia^+B220^- LN cells from UV/sensitized mice was observed (11% increase) compared to sensitized or UV treated alone (16 and 4%, respectively). This was also true of CD80 and CD86, where the combined UV/sensitized percent increase (34 and 118%, respectively) did not exceed UV treated alone (21 and 125%, respectively) plus sensitized alone (19 and 118%, respectively). An expected additive effect for the combined UV/sensitized condition was not seen either with the costimulatory molecules nor IL-12. Thus, low-dose UVB irradiation alone does not down-regulate, but rather up-regulates costimulatory molecule expression associated with IL-12 p40 in DLN. However, UV skin has a suppressed up-regulation of expression of costimulatory molecules on APC and IL-12 p40 in response to antigen.

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***In Vivo* Evidence that Epidermal Langerhans Cells are Targeted Selectively for Gene Expression by a Dectin-2 Promoter**

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Despite their unsurpassed antigen presenting capacity especially for naive T cells, Langerhans cells (LC) and dendritic cells (DC) have been rarely used as targets of gene-based techniques because well-defined regulatory elements controlling specific expression in these cells have not been identified. Previously we reported that a 5′-flanking region in the mouse dectin-2 gene (Dec2FR) directs gene expression in LC/DC (but not other cell) lines. To evaluate LC/DC specificity *in vivo*, we generated transgenic mice bearing a luciferase (Luc) reporter gene controlled by Dec2FR. In these mice, Luc activity was highest in skin, much less in lymphoid organs, and close to background level in other organs. Epidermal cells expressed markedly high activity, and depletion of Ia^+ cells (LC) almost completely abrogated such activity. By contrast, Luc activity in spleen cells was 200-fold lower than in epidermal cells, and depletion of $CD11c^+$ DC led to 30% reduction of total spleen Luc activity. Among FACS- or magnetic bead-purified cells, Ia^+ epidermal LC expressed the highest activity (12 RLU/cell); $CD11c^+$ spleen DC and Mac-1^+ peritoneal macrophages (MF) showed detectable but markedly low activity (0.08 and 0.07 RLU/cell, respectively), whereas $B220^+$ B cells and $CD3^+$ T cells exhibited background levels. Even when activated, B cells failed to express Luc activity, whereas T cells and MF showed inducible Luc activities that were still 10-fold lower than resting epidermal LC. We conclude that epidermal LC are targeted selectively for high-level constitutive gene expression by Dec2FR in Luc transgenic mice. These findings have encouraged us to use Dec2FR in developing additional mouse models of LC-targeted gene expression that will lead ultimately to an improved understanding of LC biology and cutaneous immunology, with potential applications for immunotherapy.

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Catecholamines Inhibit Epidermal Immune Reactions by Directly Affecting Langerhans Cells

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Adrenergic factors are present in the skin and a role in cutaneous immune function has been proposed. Epidermal LC are found in close vicinity to nerve endings and may thus be affected by nerve-derived catecholamines. We have previously shown that the murine skin derived dendritic cell line XS52 ($H\text{-}2^d$) and LC enriched to 98% homogeneity from CAF_1 mice ($H\text{-}2^{a/d}$) express mRNA for a1a, β1 and β2-adrenergic receptors. Furthermore we showed that epinephrine (EPI) and norepinephrine (NE) inhibit antigen presentation in three different functional assays: (a) *in vitro* EPI and NE inhibited presentation of a specific antigen by BALB/c ($H\text{-}2^d$) epidermal cells (EC) to a reactive Th1-clone in a dose-dependent manner; (b) both catecholamines also significantly inhibited EC (enriched to ~12% LC-content by antibody- and complement mediated lysis of Thy 1.2-bearing cells) presentation of tumor associated antigens for elicitation of delayed-type hypersensitivity (DTH) in previously immunized CAF_1 mice. This inhibitory action was blocked by a β-, but not an a-adrenergic antagonist and thus appears to be a β-adrenoceptor mediated process; (c) intradermal injection of EPI prior to sensitization by topical application of 2,4-dinitro-1-fluorobenzene significantly reduced the contact hypersensitivity reaction elicited 7 days later upon rechallenge with the hapten. To determine whether catecholamines affect LC antigen presenting capability directly or indirectly through an effect on other cells (e.g. cytokine release by keratinocytes), we enriched murine EC preparations by antibody- and complement-treatment followed by cell separation utilizing $I\text{-}A^d$ targeted magnetic beads. This process yielded a LC population of 98% purity. These LC were cultured for 3 h in graded concentrations of EPI and NE (10^{-7} M–10^{-9} M), pulsed with keyhole limpet hemocyanin (KLH) overnight and subsequently cocultured with the KLH-responsive T cell clone HDK1. Both EPI and NE inhibited presentation of KLH by murine enriched LC to this T cell clone in a dose dependent manner, as assessed by IFN-χ production at 72 h. These findings support the hypothesis that adrenergic agents are involved in regulation of skin immune function by directly affecting LC-antigen presenting capability.

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The Interactions of Staphylococcus aureuswith Murine Langerhans Cells

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Staphylococcus aureus is the most common cause of cutaneous infections and is present on lesions of chronic inflammatory skin diseases such as atopic dermatitis; yet, the interactions between bacteria and host are poorly understood. We investigated the ability of *S. aureus* to adhere to, invade into, and grow on Langerhans cells *in vitro*. Adherence was determined at specific time points by washing away nonadherent bacteria, disrupting cell monolayers, and performing quantitative plate assays. Intracellular bacteria were similarly quantitated after killing extracellular bacteria with lysostaphin prior to monolayer disruption (lysostaphin is cytotoxic to *S. aureus* but does not enter host cells, thus, intracellularly located bacteria are protected from its effects). *S. aureus* adhere to and invade into Langerhans cells quickly and efficiently; adherence approached 100% immediately and 50–80% of the bacteria were located intracellularly within 30 min After a stationary phase, the bacteria within the Langerhans cells replicated intracellularly. The results of this present study show that *S. aureus* adhere to, invade into, and grow on Langerhans cells at rates comparable to other macrophage cell lines while, the invasion rate is significantly higher than nonprofessional phagocytes (i.e. keratinocytes, fibroblasts, melanocytes). The skin is the first level of host defense against many pathogens. As the primary antigen presenting cells of the skin, Langerhans cells have an important role in either directly or indirectly shaping the antigen-specific host immune response to cutaneous pathogens. These studies characterize the initial interactions between Langerhans cells and bacteria, which is an important step in studying both the host immune response and the mechanisms employed by *S. aureus* during skin infections.

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The Cellular and Functional Properties of Isolated Human Langerhans Cells are Affected by Keratinocyte Coculturing

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We have recently shown that human epidermal Langerhans cells (LCs) obtained by magnetic-activated cell sorting (MACS) with optimized anti-CD1a concentrations, resemble immature intraepidermal cells and undergo only slight spontaneous maturation upon culture. This *in vitro* system thus represents an excellent tool to analyze in detail the effects of bacterial antigens and cytokines on the phenotypical and functional properties of immature LCs. Freshly MACS-isolated ultrapure LCs with high levels (95%) of HLA-DR and CD1a expression were incubated with LPS, CD40L, TNF-α, Flt3-L, TGF-β, IFN-γ and INF-ω for 24 h. Triple color flow cytometry revealed up-regulation of the CD83 expression after stimulation with LPS and TNF-α, increased CD80 expression after TGF-β, and up-regulation of CD95 ligand after IFN-γ incubation. A significant increase in the MLR with CD4-positive T cells was only observed after incubating the cells with LPS. However, none of these incubations were able to inhibit the loss of viability and decreased expression of CD1a and HLA-DR occuring upon spontaneous culturing of the cells. When coculturing freshly MACS-isolated LCs with the human keratinocyte cell line HaCaT, there were a rather constant viability, and CD1a and HLA-DR expression. This coculture system was additionally found to strengthen the effects of inflammatory stimulation. In conclusion, our findings demonstrate that (i) proinflammatory cytokines and bacterial antigens induce distinct phenotypical and functional changes of immature human LCs, and (ii) keratinocytes play a crucial role in regulating not only the viability, but also the differentiation and maturation of isolated LCs. This regulation obviously requires direct interactions of the cells, because the incubation with keratinocyte supernatants failed to affect the behavior of cultured human LCs.

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Effects of the Topical Application of Cholera Toxin on the Migration of Human Langerhans Cells

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Dendritic cells, including epidermal Langerhans cells, are potent professional antigen presenting cells (APC). The development of immunization strategies which target antigen delivery to Langerhans cells, which are readily accessible in the skin, is an attractive approach to vaccine design. In addition to antigen delivery, effective cutaneous vaccination strategies will likely induce the activation and migration of resident dendritic cells, typically through the use of adjuvants. Applying cholera toxin (CT), which has been shown to have potent adjuvant effects, onto intact skin has been proposed to induce migration, trafficking, and/or activation of Langerhans cells. Here, we investigate the migration of Langerhans cells out of human epidermis in response to the topical application of CT. We compared migration of Langerhans cells from human foreskins after topical application of CT, a model protein antigen alone (fluorescent-OVA (DQ-OVA)), the combination of CT and antigen, or vehicle alone as a control. Migration of Langerhans cells was determined by the quantification of HLA-DR positive cells remaining in epidermal sheets at various time points. Our results indicate a significant increase in migration of Langerhans cells out of skin exposed to either CT or DQ-OVA at 3 h, compared to vehicle control. In comparison studies, the difference between migration induced by CT alone, simultaneous application of CT and DQ-OVA, or DQ-OVA alone was not significant. Furthermore, the majority of LC migration occurred within the first 3 h, and increasing contact time between the adjuvant/antigen and the skin (3 h vs. 28 h exposure) did not appear to significantly enhance LC migration. These results indicate that while CT induced LC migration, the migration observed was not significantly greater than administration of the antigen DQ-OVA alone, and that only a brief exposure of either of these agents is sufficient to induce LC migration from human skin.

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Enhanced Internalization and Presentation of Transport Peptide Conjugated Antigens by Dendritic Cells

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We have examined the effectiveness of an antigen delivery strategy that utilizes a transport peptide (PTD-5) to deliver antigen to dendritic cells. First, we compared the internalization of FITC-OVA conjugated to PTD-5, to the control antigens FITC-OVA and FITC-OVA conjugated to Avidin, by bone marrow-derived dendritic cells grown for 5 days in the presence of GM-IL4. Compared to controls, accumulation of FITC-OVA-PTD-5 was dramatically increased in the DCs after as little as 15 min of incubation. A large proportion of the intracellular FITC-OVA-PTD-5 appeared to be concentrated in many large compartments colocalizing with the lysosomal marker, Lamp-1. Inhibition studies suggest that the internalization process involves not only an endocytic pathway, but also a possible direct translocation through the membrane. In contrast, FITC-OVA or FITC-OVA-Avidin was taken up by cells with less efficiency, and only through the endocytic pathway. To evaluate the efficiency of presentation of PTD-5 conjugated antigens, the capacity of DCs to present conjugated or control antigens to OVA-specific MHC class I and class II restricted hybridomas was determined *in vitro*, and the capacity of antigen pulsed BMDCs to present antigen to MHC class I-restricted, ovalbumin-specific, CD8+ T cells (OT-I cells) was evaluated *in vivo*. These experiments demonstrated that presentation of OVA-PTD-5 was significantly more efficient through both MHC class I and II processing pathways both *in vitro* and *in vivo*. Our data clearly indicate that PTD–5 association with antigen facilitates active and passive antigen entry into BMDCs, and enhances MHC class I and II antigen presentation by BMDCs *in vitro* and *in vivo*. This approach for delivering antigen to dendritic cells may be an important strategy for the development of vaccines and immunotherapies.

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Dendritic-Cell Based Immunotherapies: Route of Administration Effects Dendritic Cell Trafficking and Regional Immune Responses

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Dendritic cells (DCs) are the most potent antigen presenting cells (APCs). DC vaccines, based on the adoptive-transfer of antigen-loaded DCs, are currently being evaluated as a strategy to induce antitumor immunity in cancer patients. The effects of different routes of administration of DCs on DC trafficking, *in vivo* localization, and immunogenicity has not been conclusively determined, and remains a critical issue for the development of DC-based therapies. To address this issue, the effect of different routes of administration on subsequent localization of DCs in lymphoid organs, and the correlation between DC localization and immunogenicity was determined. Identical numbers of BmDCs were fluorescently labeled and injected into C57BL/6 mice either s.c. or i.v. Lymphatic organs were harvested at varying time points after immunization and the presence of labeled DCs in the lymph nodes and spleen was determined by flow cytometery and quantitative morphometric analysis. After s.c. injection, labeled DCs were detected in the regional but not distant lymph nodes or spleen. Labeled cells were present in the regional lymph nodes for at least 5 days, reaching a peak by day 2. After i.v. injection labeled DCs were found in spleens, but not in lymph nodes during the first 3 days. To evaluate regional immunogenicity, we made use of an assay based on the adoptive transfer of CFSE fluorescent dye-labeled OT-1 CD8+ T-cells into naïve syngeneic recipients to measure antigen-specific T-cell proliferation *in vivo*. After 3 days, considerable antigen-specific proliferative responses were observed in the draining lymph nodes, but not the spleens, of SC injected animals. Conversely, IV injection of DCs led to brisk antigen-specific proliferative responses in the spleens, but not in the lymp nodes, of injected animals. Taken together, these results demonstrate that the route of administration effects subsequent trafficking and localization of adoptively transferred DCs, and influences the development of regional immune responses.

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Dendritic Cells (DC) Treated with TAT Protein Transduction Domain (PTD)-Containing Recombinant Tumor Antigens Efficiently Induce Cytotoxic Lymphocytes (CTL) and Tumor Immunity

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There is considerable interest in harnessing the antigen presenting power of DC to elicit CTL in cancer patients. CD8+ CTL recognize peptides that preferentially derive from endogenous proteins and bind to MHC class I. We hypothesized that protein transduction could be used as an alternative to cDNA transfection or viral infection to introduce proteins into DC and to load MHC class I with peptide epitopes of interest. Plasmids encoding full length chicken ovalbumin (Ova) with His6 and HA tags were engineered with and without an 11 amino acid HIV TAT PTD, and bacterial recombinant proteins were purified. TAT-HA-Ova rapidly entered EL-4 lymphoma cells, while PTD-deficient HA-Ova did not. TAT-HA-Ova, but not HA-Ova, also sensitized EL-4 thymoma cells to lysis by CTL specific for the Ova SIINFEKL peptide epitope. SIINFEKL:H-2Kb complexes were detected on TAT-HA-Ova transduced EL-4 cells and transduced bone marrow DC (BMDC) with a mab (25.d1.16). Subq injection of TAT-HA-Ova pulsed BMDC led to generation of Ova-specific CTL in both normal and CD4+ T cell-deficient mhc class ii knockout mice. Bmdc pulsed with HA-Ova or the control protein TAT-HA-β-Gal did not induce CTL. Vaccination with TAT-HA-Ova pulsed bmdc prevented growth of Ova CDNA EL-4 stable transfectants into tumors in 24 of 26 recipients. Twenty of 30 mice that received SIINFEKL peptide pulsed DC were also protected, while recipients of untreated or TAT-HA-β-Gal pulsed BMDC were not. Recombinant TAT PTD-containing protein antigens can be easily prepared, readily enter DC and are processed for MHC class I presentation as if they were synthesized endogenously. DC transduced with ptd-containing tumor antigens are also potent inducers of ctl that can efficiently eradicate tumors in mice. This vaccination strategy has advantages over those that are in current use, and may promote development of efficacious dc-based immunotherapies for use in patients.

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Induction of Tumor-Specific Protective Immunity by *In Situ* Langerhans Cell Vaccine

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Many dendritic cell (DC)-based vaccine protocols have been recently developed for the treatment of cancer patients. However, time- and cost-consuming "customizing" processes that are required in the current format for isolation, expansion, and antigen-pulsing of autologous DC remain a major hurdle for preventing broader clinical applications of this new vaccine concept. Here we report a novel format that requires no *ex vivo* DC manipulation. Upon exposure to haptens, Langerhans cells (LC) migrate to draining lymph nodes (DLN) and begin to exhibit features of mature DC. We sought to entrap these migratory LC by creating an artificial chemokine gradient in their homing path. Among several chemokines tested in the transwell system, MIP-3β promoted optimal migration of mature DC. Based on these observations, we formulated ethylene-vinyl-acetate (EVA) polymer rods that released biologically intact MIP-3β in a controlled fashion (300 ng per ml release in the first 48 h). When these rods were implanted subcutaneously into mouse abdomen and DNFB was applied over the implantation sites, marked LC accumulation was observed around the implanted MIP-3β rods, but not control BSA rods, and only after DNFB painting. FITC-triggered LC homing to DLN (assessed by counting $IA^+/FITC^+$ LN cells) was markedly (80%) inhibited by MIP-3β rod implantation at 24 h. Recovery of $IA^+/FITC^+$ LN cells increased thereafter to the normal level at 72 h, indicating that LC can be entrapped transiently without affecting their homing potential. As an attempt to load migratory LC with tumor-associated antigens (TAA) at the entrapment sites, we coimplanted MIP-3β rods together with the second EVA polymer rods releasing ovalbumin (OVA to serve as a model TAA) and applied DNFB 24 h later. These mice developed potent CTL activities to lyse the OVA-transduced E.G7-OVA tumor target, but not the parental EL4 tumor target, and exhibited almost full protection against subsequent challenge with E.G7-OVA tumor cells ($p < 0.01$, 10 mice/panel). Only marginal, if any, CTL activities or protection were observed in control panels receiving MIP-3β rod alone, OVA rod alone, or both rods but in separate locations. Thus, tumor-specific protective immunity is readily inducible by combining MIP-3β-mediated LC entrapment and EVA polymer-based LC loading with relevant TAA. Our *in situ* LC vaccine strategy represents a major breakthrough moving the DC-based vaccine concept toward practical medicine.

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Suppression of Allogeneic Immune Responses by CD95L-Transduced Killer Hybrids Created by Fusing Donor- and Recipient-Derived Dendritic Cells

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Allogeneic immune responses, which are initiated by dendritic cells (DC) of both donor and host origins, remain a major obstacle in organ transplantation. Allo-antigens are presented by dual mechanisms: (a) *direct* presentation, in which T cells recognize intact allo-MHC molecules expressed on allogeneic DC; and (b) *indirect* presentation, in which T cells recognize allogeneic peptides presented by syngeneic DC. Here we report a novel strategy designed to suppress both pathways. The stable DC line XS106 (A/J mouse origin) was transfected with CD95L cDNA and fused with splenic DC purified from allogeneic BALB/c mice. The resulting "killer" DC-DC hybrid clones expressed CD95L and MHC class I and class II molecules of both A/J and BALB/c origins and otherwise maintained phenotypic features of mature DC. In primary allo-MLR, killer DC-DC hybrids completely abrogated proliferative responses of both $CD8^+$ and $CD4^+$ T cells from A/J mice to BALB/c-derived DC, as well as T cell responses in the reversed direction (BALB/c→A/J). In the ^{3}H-thymidine release assay, killer DC-DC hybrids efficiently induced apoptosis of allo-reactive T cells of both A/J and BALB/c origins. On the other hand, CD95L-transduced XS106 DC (i.e. killer DC expressing A/J-derived MHC molecules alone) suppressed the allo-responses between the two strains only partially, corroborating our previous report (*Nature Med* 5:930, 1999). Vector-transfected control DC-DC hybrids delivered potent activation signals, instead of apoptotic signals, to allo-reactive T cells from either strain. These results document the unique ability of killer DC-DC hybrids to suppress complex allo-responses that occur bi-directionally and via direct and indirect presentation mechanisms. When i.v. injected into BALB/c mice, killer DC-DC hybrids inhibited almost completely their delayed-type hypersensitivity (DTH) responses to A/J-associated allo-antigens, but not to irrelevant allo-antigens of C57/BL6 origin. Killer DC-DC hybrids also suppressed DTH responses of A/J mice to BALB/c-associated allo-antigens, again, indicating bi-directionality. Moreover, the onset of GVHD in (BALB/c × A/J) F1 hosts receiving A/J-derived hematopoietic cell transplantation was suppressed significantly ($p < 0.001$) by killer DC-DC hybrid treatment. These results establish a new concept that allogeneic immune responses can be prevented by CD95L-transduced hybrids created by fusing donor- and host-derived DC.

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BAFF, a TNF Family Member Expressed by Dendritic Cells, is Involved in T Cell Activation

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BAFF is a recently described TNF family member with a potent stimulatory activity on B cells. BAFF is expressed in dendritic cells and we will present data on the T cell costimulatory activity mediated by BAFF. BAFF induces T cell proliferation and IFNg secretion in the presence of suboptimal concentrations of antibodies to the TCR/CD3 complex. Interestingly, this costimulation pathway is additive to the CD28 pathway. Resting T cells (CD4+ and CD8+) as well as primed T cells were found to be responsive to this costimulation pathway. This indicates that BAFF may not only regulate humoral but also cellular immune responses.

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Papillomavirus-Like Particles Cause Dendritic Cell Maturation and Induce a Potent *In Vitro* Primary Immune Response

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High risk human papillomaviruses (HPV) are present in virtually all cases of cervical carcinomas worldwide providing an opportunity to prevent a major cause of cancer deaths in women through vaccination. Papillomavirus-like particles (VLPs), composed of L1 major capsid proteins, are potent inducers of humoral and cellular immune responses, making them attractive vaccine candidates and early phase clinical trials are currently under way. However, the mechanisms of immune activation by VLPs remain to be elucidated. Here we focused on the role of dendritic cells (DCs) in VLP-induced immunity, given their essential role in initiating and modulating T cell responses. We found that murine bone marrow derived dendritic cells (BMDCs) effectively bound and rapidly internalized bovine papillomavirus (BPV) VLPs. Exposure to fully assembled VLPs of BPV, human papillomavirus (HPV)16 or HPV18, but not to predominately disordered HPV16 capsomers induced acute phenotypic maturation of BMDCs. Interestingly, structurally similar polyomavirus VLPs bound to the DC surface and were internalized, but failed to induce maturation. DCs that had incorporated HPV16 VLPs produced proinflammatory cytokines IL-1β, IL-6 and TNFα. Production of IL-12p70 by VLP-exposed DCs required the addition of syngeneic T cells or recombinant IFNγ. Finally, BMDCs pulsed with HPV16 VLPs induced Th1-dominated primary T cell responses *in vitro*. Our data provide evidence for the ability of DCs to respond to virion structural patterns thereby causing a rapid transition from innate to specific immunity. They furthermore offer a mechanistic explanation for the striking ability of papillomavirus VLP-based vaccines to induce potent T cell responses in the absence of adjuvant.

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Mouse Bone Marrow-Derived Dendritic Cells Do Not Effectively Cross-Present Haptens and Soluble Proteins on MHC Class II Molecules

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The phenomenon of "cross-presentation" results when antigen presenting cells (APC) take up exogenous antigens and present them to CD8+ cells in the context of APC MHC class I molecules. Cross-presentation is thought to play a role in the maintenance of tolerance, in the rejection of transplants or tumor cells and in dendritic cell (DC)-based vaccines. Although cross-presentation of MHC class I-restricted antigens is well documented, much less is known about cross-presentation of antigens on MHC class II molecules. Our purpose was to determine whether there is cross-presentation on MHC class II molecules during protein and hapten sensitization. Thus, we functionally characterized mouse bone marrow-derived DC (BMDC) *in vitro* (in protein-antigen and hapten-specific assays) and *in vivo* (injecting soluble protein- and hapten-pulsed DC). For immunization purposes, $3–5 \times 10^5$ mature BMDC pulsed with 1 mg per ml hen egg lysozyme (HEL) or with 1 mM TNBS were injected subcutaneously. Using an *in vitro* T cell proliferation assay and *in vivo* delayed-type hypersensitivity (DTH) and contact sensitivity (CS) assays we found that protein- and hapten-pulsed BMDC were able to sensitize syngeneic but not allogeneic recipients. We also found that even large numbers of heat-killed allogeneic protein-pulsed or hapten-coupled BMDC (up to 10^7 cells) did not induce CS or DTH. Furthermore, if we injected BALB/c and C57BL/6-derived HEL-pulsed BMDC into F1 mice, specific secondary proliferation of primed T cells occurred only when antigen-pulsed stimulator cells syngeneic to the injected BMDC were used. These results demonstrate that, although immature DC take up and cross present particulate antigen in the context of MHC class I molecules, BMDC do not effectively cross present haptens and soluble proteins on MHC class II molecules. These findings increase our understanding about the potential *in vivo* immunogenic effects of BMDC when they are used in vaccination and immunotherapy protocols.

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Transfection of Human Skin Dendritic Cells by Biolistic Delivery of Naked DNA

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Gene therapy techniques can be important tools for the induction and control of immune responses. Antigen delivery is a critical challenge in vaccine design, and DNA based immunization offers an attractive method to deliver encoded transgenic protein antigens. In the present study, we used a gene gun to transfect human skin organ cultures with a particular goal of expressing transgenic antigens in resident cutaneous dendritic cells with plasmid DNA encoding for different reporter proteins (EGFP or firefly luciferase) or for the human melanoma antigen MART-1. Particle delivery to DC was determined in the epidermis by confocal microscopy and in migratory DC (miDC) by transmission electron microscopy. The expression of reporter proteins was determined by confocal microscopy and luciferase assays in epidermal or dermal sheets as well as in purified epidermal LC or in miDC. Activation and migration of LC was analyzed at different time points after gene delivery. The possibility of direct transfection of cutaneous DCs was analysed by RT-PCR. The ability of transfected LC to present transgenic peptides and to stimulate a CTL response was analysed in cocultures of LC transfected with a pCDNA encoding for the human melanoma antigen MART-1 with specific CTL clone HLA-A2 restricted. Our studies demonstrate that delivered gold particles are observed primarily in the epidermis, even when high helium delivery pressures are used. We demonstrate that Langerhans cells resident in the basal epidermis can be transfected, and that biolistic gene delivery is sufficient to stimulate the activation and migration of skin dendritic cells. RT-PCR analysis of dendritic cells, which have migrated from transfected skin, demonstrates the presence of transgenic mRNA, indicating direct transfection of cutaneous dendritic cells. Importantly, transfected epidermal Langerhans cells can efficiently present a peptide derived from the transgenic melanoma antigen MART-1 to a MART-1 specific CTL.

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Comparative Analysis of Strategies Designed to Deliver Tumor-Derived Proteins to Dendritic Cells

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Dendritic cells (DCs) are potent professional antigen presenting cells capable of inducing effective antigen-specific CTL-mediated antitumor immunity. The development of effective strategies for delivering tumor antigen to DCs *in vitro* and *in vivo* is critical for the development of antitumor immunotherapies. In addition to the challenge of Ag delivery, most current tumor immunization strategies depend on the identification and production of appropriate tumor Ags. The development of strategies to deliver tumor-derived proteins into the appropriate antigen presentation pathways of dendritic cells could potentially overcome this limitation, resulting in polyvalent immunization to multiple tumor-specific epitopes, while obviating the need to identify specific tumor Ags. Our laboratory and others have investigated the use of whole tumor cells or tumor cell lysates as a source of tumor antigens for DCs. While both methods result in the presentation of tumor antigens by DCs, little has been done to compare their relative efficacy. Here, we use fluorescence microscopy and flow cytometry to compare the transfer of tumor-derived proteins to DCs following coculture with either intact tumor cells or tumor cell lysates. Our results demonstrate that culture with either whole tumor cells, or tumor cell lysates, results in the transfer of tumor-derived proteins to DCs. Quantitatively, feeding tumor cell lysates derived from flourescently labeled tumor cells results in a significantly greater increase in mean fluorescence intensity of DCs when compared to the fluorescence intensity of DCs cocultured with intact, identically labeled tumor cells. These results indicate that these distinct methods of tumor antigen delivery to DCs result in significant differences in the efficiency of antigen delivery, which may impact the functional immunogenicity of tumor-antigen loaded DCs.

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The Skin Receiving Gene Therapy Induces Immune Response through Cross-priming

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Although a current theme of gene therapy is mostly focused on how to transfer the gene into target tissues, another important subject is to control immunologic reaction to newly introduced molecule. Here we study the immune response of the host after gene transfer into the epidermis, by means of establishing K5-mOVA, a transgenic mouse expressing a membrane-bound form of ovalbumin (OVA) under keratin5 promoter as a model. To represent the situation that patients receive autologous skins having been manipulated with the gene, the skin expressing OVA is transplanted on nonexpressing syngeneic mice. The skin graft is, however, rejected, implying that the immune system of the host recognizes the newly synthesized protein coded in the gene. To analyze the recognition mechanism of $CD8^+$ T cells that play a crucial role in the rejection of endogenously expressed antigen, OVA-specific $CD8^+$ T cells are transferred into K5-mOVA mice. OVA specific $CD8^+$ T cells home to the skin draining lymph node, followed by being activated and proliferating. This response that is restricted with MHC class I antigen of bone marrow derived cells is referred to cross-priming. From these results, we propose that the gene should be modified to lessen the immunogenecity of the product otherwise the immune response of the host might bring the gene therapy to no vail.

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Modulating Specific Immune Responses by Varying the Route of Vaccine Administration

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We have previously shown that the subcutaneous (s.c.) injection of a vaccine consisting of β-galactosidase and poly L-arginine induces an antigen-specific T cell response able to protect animals against a challenge with tumor cells expressing this model antigen. In this study, we asked whether the route of administration would influence quality and quantity of this response. To this end, mice received the protein vaccine either via the s.c. or intradermal (i.d.) route and were then analyzed for the occurrence of antitumor immunity. We found that, while the s.c. administration of the protein vaccine prevented the growth of β-gal–expressing tumor cells (RENCAlacZ) in only 50% of the animals, the i.d. application of the antigenic complex resulted in protection rates of 80–100%. Depletion experiments revealed that CD8+ T lymphocytes are crucial in this regard. β-gal-specific antibodies present in these animals did probably not contribute to the antitumor effect observed as they failed to bind intact RENCAlacZ cells. To test whether the protection rates of the two routes of protein vaccine administration would correlate with the strength of the T cell response, we quantified the number of cytokine-producing CD8+ T lymphocytes recognizing the immunodominant epitope of β-gal. While both modes of vaccine application activated similar numbers of IL-4-producing T cells, the i.d. vaccine administration was superior in its capacity to elicit IFNγ-expressing T lymphocytes. These results demonstrate that the i.d. administration of surrogate tumor antigens induces a more effective state of antitumor immunity than the vaccination via the s.c. route. Our findings also lend further support to the hypothesis that the Th1/Tc1 rather than the Th2/Tc2 component of the immune response is of decisive importance for the prevention of tumor growth.

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CpG Motifs Act as Efficient Adjuvants for pDNA-Based Vaccines to Induce Protective Antitumor T Cell Responses only when Directly Introduced into Vector Sequences

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DNA sequences containing a consensus immunostimulatory motif, so called CpG motifs, have been shown to stimulate induction of cellular and humoral immune responses. In this study we asked whether CpG motifs are a useful adjuvant for genetic cancer vaccines to induce antigen-specific T cell responses *in vivo*. We therefore analyzed the induction of protective T cell responses against the melanoma self-antigen Pmel17/gp100 in the experimental Cloudman M3/DBA/2 mouse melanoma model. When given simultaneously as oligodeoxynucleotides, CpG motifs abrogated any protective effect induced otherwise by mPmel17-encoding pDNA against a tumor cell challenge with Pmel17 ± M3 melanoma cells. To analyze the adjuvant activity of CpG motifs present in vector sequences, we cloned 16 CpG motifs into the backbone sequences of pDNA vector pUK21-A2 thereby generating its CpG-enriched derivative pMCG16. Mice immunized with mPmel17/pMCG16 pDNA showed a significantly increased protection against subsequent melanoma challenge with $Pmel17^+$-M3 melanoma cells as compared to mice immunized with the parental vector. Induction of an antigen-specific T cell imune response by mPmel17/pMCG16 pDNA could be demonstrated by (i) induction of protection against $Pmel17^+$- but not $Pmel17^-$-M3 melanoma sublines (ii) complete loss of protection by *in vivo* $CD4^+/CD8^+$ T cell but not NK cell depletion and (iii) the detection of a Pmel17/gp100-specific T cell response in CTL assays at levels consistently higher than in splenocytes immunized with the parental vector construct. These results demonstrate for the first time that CpG motifs can improve the induction of protective, antigen-specific T cell IR by pDNA vaccines when introduced into vector sequences but not when coadministered as oligodeoxynucleotides.

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DNA-Based Vaccines: Mechanisms of Immune Activation

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We have recently shown that the direct intradermal (i.d.) injection of plasmid DNA coding the autologous form of Pmel17 induces a T cell response to this antigen that is able to protect DBA/2 mice against a challenge with an otherwise lethal dose of syngeneic Pmel17-expressing melanoma cells. In the present study, we attempted to explore the mechanism(s) of this protective response. To this end, we used β-galactosidase (β-gal) as a surrogate antigen. A single i.d. injection of a VR1012 vector engineered to express the lacz gene led to the activation of β-gal-specific CTLs and antibodies (IgG2aIgG1). Mice depleted of CD4+ T cells failed to mount a specific CTL as well as antibody response. To learn more about the fate of the vector at the injection site (i-site), we injected BALB/c mice with VR1012 lacz, excised the i-sites at defined time points thereafter (days 1, 4, 6, 8, 11, 13) and evaluated the animals for the presence of β-gal-specific T and B cells on day 14. Results obtained showed that the removal of the i-site until d 6 prevented the activation of peptide epitope-specific T cells (as assessed by Elispot) and of a specific antibody response (as assessed by ELISA). We began to detect β-gal-specific immunity when the i-site stayed *in situ* for 8 days (number of Elispots 20% of those seen in nonexcised control mice). By day 11, the immune reaction was approximately half as strong as that seen in nonexcised controls and comparable to it by day 13. Our data suggest that for the formation of the sensitizing antigenic moiety, the intradermally injected vector must interact with skin cells rather than being immediately transported to the regional lymph node. The experimental system presented in this study should not only be useful to identify the critical sensitizing cell population of the skin but also to devise and test strategies to improve the transfection efficacy and thereby the immunologic potency of DNA-based vaccines.

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Comparison of Genetic with Conventional Protein Vaccines: Induction of Protective Antitumor T Cell Responses Against Tumor Antigen Expressed by pDNA-Based Vaccine but not Against Recombinant Protein

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The recent development of genetic vaccines raises the question whether these genetic vaccines are superior to conventional immunization strategies employing the respective protein for the induction of protective T cell responses. We therefore cloned the 1.9 kb full length cDNA murine homologue of human MAA Pmel17/gp100, generated a recombinant baculovirus containing the respective cDNA sequence by ligation independent cloning, expressed the recombinant protein in Sf9 insect cells, and confirmed its identity by SDS-PAGE and Western blot analysis. This recombinant full-length protein was compared with a pDNA vaccine for the induction of protective antigen-specific T cell responses in the experimental Cloudman M3/DBA/2 mouse melanoma model. Mice genetically immunized with mPmel17-encoding pDNA showed significant protection against subsequent tumor challenge with $Pmel17^+$-M3 melanoma cells in contrast to mice immunized with the recombinant protein ($p < 0.001$ at day 15). Whereas induction of an antigen-specific T cell response by mPmel17-encoding pDNA could be demonstrated by (i) induction of protection against $Pmel17^+$- but not $Pmel17^-$-M3 melanoma sublines (ii) complete loss of protection by *in vivo* $CD4^+/CD8^+$ T cell depletion and (iii)) the detection of a Pmel17/gp100-specific T cell response in CTL assays, antigen-specific T cell responses could not be detected in mice immunized with the respective recombinant protein. These results demonstrate for the first time that genetic immunization with antigen-encoding pDNA is superior to immunization with the respective recombinant protein for the induction of antigen-specific, protective antitumor T cell responses *in vivo*.

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Suppression of the Induction of Delayed-Type Hypersensitivity (DTH) to Tumor-Associated Antigens by Intravenous Administration of Tumor RNA can be Transferred with Spleen Cells

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Intravenous (i.v.) administration of protein antigens can induce relative tolerance to subsequent immunization with that antigen. We have previously reported that i.v. injection of total cellular RNA from the S1509a spindle cell tumor line also reduces immunity to tumor associated antigens derived from this tumor cell line. In this study we examined whether the suppression of immunity can be transferred by spleen cells. A group of CAF1 mice ($n = 10$) was primed i.v. with 100 μg of total cellular RNA from the S1509a spindle cell tumor line, control groups were primed i.v. with saline alone or with unrelated RNA from the NS cell line. One week after i.v. injection the spleen cells of the mice were harvested and subsequently transferred i.v. into naïve CAF1 mice. All mice were then immunized by subcutaneous injection of disrupted S1509a cells 3 times at weekly intervals. One week after the last immunization, syngenic epidermal cells enriched for Langerhans cell content (eEC) by antibody- and complement-mediated deletion of Thy-1-bearing cells were pulsed with a soluble extract of the S1509a cells as a source of tumor-associated antigens (TAA). TAA-pulsed eEC were then injected into a footpad of each mouse. Twenty-four hour footpad swelling was assessed as a measure of DTH with a spring-loaded micrometer. Mice given spleen cells from S1509a RNA-primed mice demonstrated a significantly ($p < 0.001$) smaller DTH response (31×0.01 mm ± 1.9 [SEM]) compared to mice given spleen cells from mice primed i.v. with saline (49×0.01 mm ± 2.4 [SEM]) or unrelated NS-RNA (43.3×0.01 mm ± 1.9 [SEM]). These results demonstrate that the suppression of the DTH response to S1509a derived TAA induced by i.v. administration of total cellular RNA from the S1509a spindle cell tumor line can be transferred with spleen cells.

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Epicutaneous Application of Peptide Allows Priming of Cognate Cytotoxic T Cells in the Presence of IL-12 and in the Absence of Barrier Disruption

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It has recently been shown that epicutaneous application of peptide to tape stripped skin can allow priming of cognate cytotoxic T cells (CTL). Barrier disruption results in increased permeability to exogenous peptides and in the activation of resident dendritic cells. IL-12 is a cytokine that is known to facilitate the priming of CTL and can be produced by keratinocytes. The purpose of this study was to determine whether the epicutaneous administration of peptide results in the priming of cognate CTL in the absence of barrier disruption. Using a patch protocol, we immunized mice with a peptide (SIYRYYGL) recognized by the 2C T cell receptor on Kb. Naïve T cells transgenic for the 2C T cell receptor were adoptively transferred to these animals prior to immunization. The presence of systemically administered IL-12 (1 mg IP daily, on days 0–2) allowed the proliferation of cognate CTL as determined by FACS analysis. Further, these cells were functional as determined by a standard chromium release killing assay. Thus epicutaneous administration of peptide to intact skin can result in the priming of CTL in the presence of IL-12. Strategies that enhance local IL-12 release may potentiate CTL priming to epicutaneous antigen.

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Delayed Type Hypersensitivity Skin Reactions to HIV-1 Epitopes Induced with Engineered Plasmids in the Guinea Pig

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We have found that delayed type hypersensitivity (DTH) to protein can readily be induced in the guinea pig with a plasmid expressing that protein. In a typical experiment three guinea pigs were sensitized by the intradermal injection of 100 micrograms of a plasmid expressing gp160 of the HIVmn envelope protein, in PBS. Ten days later they and three naive guinea pigs were challenged intradermally with gp120 protein. The perpendicular diameters in mm. of the induration of the skin tests at 24 h of the plasmid sensitized guinea pigs was 13×15, 12×12 and 10×13, and that of the controls was 0×0, 0×0 and 0×0. The histology of the skin reactions showed a round cell infiltrate and edema characteristic of DTH. Other experiments with plasmids expressing unrelated proteins demonstrated the specificity of the sensitization. In further studies, we sensitized guinea pigs to HIV-1 envelope with plasmids, or with protein in complete Freund's adjuvant. Epitope analysis by skin testing first with pools and then with individual peptides from a group of overlapping peptides encompassing gp120 revealed common epitopes as well as epitopes unique to each method of sensitization. The model provides a useful technique for rapidly comparing different vaccine strategies and for optimizing immunization programs as relates to cell mediated immunity.

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Treatment of Human Cells with HSP70-Rich Yeast Extract Enhances Cell Thermotolerance and Resistance to Stress

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Heat Shock Proteins (Hsp) represent a family of widely expressed chaperon proteins implicated in maintaining cell integrity after an exposure to a stress stimulus. Hsp expression is regulated at the transcriptional level by Heat Shock Factors (HSF) and an overexpression is observed a few hours after stress exposure. Based on the great sequence homology of 70% to 85% with human Hsp, and in order to provide the cell with Hsp molecules without stress, yeast heat-induced Hsp70 and HSF extract were prepared by Biotechnology from an ascomycota fungus strain and administered to the cells in these studies. Dose studies were conducted on human fibroblasts incubated with or without the yeast extract during various points over time. Total protein extract as well as total RNA were subsequently prepared. Proteins were submitted to SDS-PAGE and immunoblotting and the expression of Hsp70 was examined. In parallel, Hsp70 mRNA level was studied using a digoxigenin-labeled probe in Northern blot experiments. Immunoblotting studies demonstrated that fibroblasts treated with the yeast extract show a very rapid increase (within 30 min) in their Hsp70 content (over their HSC level). The Northern blot experiments revealed a transient and reproducible Hsp70 mRNA expression increase 3–5 h after the incubation with the yeast extract. This suggests that the early increase in Hsp is due to yeast Hsp. Additional studies show that cells treated with yeast Hsp demonstrate no sign of cell stress and a significant increase in their thermal and UV tolerance. The cells exhibited an immediate superior resistance, with no time needed for Hsp synthesis. Studies on the *ex vivo* model confirmed this beneficial effect. These interesting results provide the first evidence that Hsp70 extract stress-free administration to the cells enhances their protection and defense from stress.

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Hyperproliferative Activity of Mast Cells Derived from Atopic Dermatitis Model Mice NC/Nga through Enhanced Tyrosine Phosphorylation of JAK/STAT Pathways

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NC/Nga (NC) mice, a model mouse for human atopic dermatitis (AD), kept in air-uncontrolled conventional circumstances spontaneously suffer from AD-like eczema and pruritus. The skin lesions of NC mice with dermatitis show hyperkeratosis, lymphocyte and eosinophil infiltration, and marked increase of mast cells, and the severity of dermatitis well correlated with the number of dermal mast cells. In this sutdy, we investigated the mechanism of mast cell hyperplasia in NC mice by using bone marrow-derived cultured mast cells (BMMC). The BMMC of NC mice showed higher uptake of [3H]thymidine by stimulation of mast cell growth factors including IL-3 and stem cell factor (SCF) than those of BALB/c mice. There was no significant difference in the expression of the specific receptors for IL-3 and SCF on mast cells between these two mice, however, tyrosine phosphorylation of Janus kinases (JAK) 2 and signal transducer and activator of transcription (STAT) proteins in postreceptor signaling of IL-3 and SCF stimulation was markedly enhanced in NC BMMC. Thus, it is suggested that the hyperactivation of JAK/STAT pathways of mast cells in response to IL-3 and SCF may be attributable to marked increase of mast cell number of the skin lesions in NC mice.

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The Secretory Mediator Release of NC/Nga Mice Mast Cells

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NC/Nga Mice, an animal model for human atopic dermatitis (AD) spontaneously suffer from AD-like eczema and pruritus associated with high serum IgE when raised in a nonsterile environment. Skin lesions of NC/Nga mice show hyperkeratosis, lymphocyte and eosinophil infiltration and marked increase of mast cells, and the severity of dermatits well correlate with the number of dermal mast cells. In this study, we investigated the degranulation response of NC/Nga mice mast cells when stimulated through the IgE receptor. First, we observed the IgE-mediated degranulation of bone marrow-derived mast cells and of peritoneal mast cells in NC/Nga, using BALB/c mice as a control, by β-hexosaminidase release assay. Although there were no significant differences in the percent release of β-hexosaminidase, the amount released and the cell content was greater in NC/Nga mice. Next, the percutaneous anaphylaxis (PCA) reaction was observed. PCA reaction was greater in NC/Nga mice compared to BALB/c mice. These results suggest that the amount of secretory granules such as β-hexosaminidase, released, may be a factor which cause its strong PCA reaction, and may contribute to the skin inflammations characteristic of NC/Nga mice.

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Fetal Skin-Derived Cultured Mast Cells (FSMC); a Novel Model to Study Cutaneous Mast Cells

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We describe a novel culture system for generating large numbers of murine skin-associated mast cells and distinguish their characteristics from bone marrow-derived cultured mast cells (BMMC). Addition of IL-3 and SCF to day 16 fetal skin single cell suspensions allowed expansion and maturation of mast cells in the presence of stromal cells. The average yield of mast cells after two weeks was 7.3 million cells per fetus at a purity of 96%. These fetal skin-derived cultured mast cells (FSMC) increased their histamine content in a time-dependent manner to 3.6 pg per cell after two weeks and 6.7 pg per cell after four weeks. Phenotypic analyses revealed much greater expression of DX5 and CD81 and lesser expression of CD77 and CD102 on FSMC as compared with BMMC. These findings suggest a close similarity between FSMC and freshly isolated cutaneous mast cells. Connective tissue mast cell characteristics of FSMC were evidenced by (1) their greater histamine content (2) the presence of heparin, and (3) their degranulation in response to compound 48/80 and substance P. Importantly, we also identified functional differences between FSMC and BMMC including (1) different dose–response curves (of IgE and antigen) after FceRI crosslinking, and (2) altered cytokine secretion (more IL-13 but much less MIP-1β and IL-6 by FSMC). Thus FSMC have many characteristics distinct from BMMC and can be used as a model of cutaneous mast cells to discern their functions and to potentially serve for screening of skin mast cell-modifying drugs.

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CD4+ T-Cells Augment DMBA Skin Carcinogenesis

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Polyaromatic hydrocarbons (PAHs) are ubiquitous environmental agents, which, when applied topically, produce cutaneous malignancies in animal models and in humans. Studies from our laboratory have shown that topical application to mice of several different PAHs also results in the development of antigen specific cell-mediated immunity. The objective of this study was to characterize the T-cells that are responsible for PAH contact hypersensitivity and to determine whether they participate in PAH-induced skin tumorigenesis. Topical application of the carcinogenic PAH, 7,12-dimethylbenz(a)anthracene (DMBA) to C3H/HeN mice, as expected, resulted in the development of contact hypersensitivity. *In vivo* administration of anti-CD8 antibodies prior to sensitization abrogated the response. In contrast, *in vivo* depletion of CD4+ T-cells with anti-CD4 antibodies augmented DMBA contact hypersensitivity and treatment of CD4+ knockout mice on a C3H background with DMBA also was found to increase DMBA contact hypersensitivity. When ELISAs were performed on T-cells from DMBA contact sensitized mice to determine the cytokine profile of T-cells, CD8+ T-cells were found to produce interferon γ and little or no IL-4, whereas CD4+ T-cells were found to elaborate both IL-4 and interferon γ. To determine if CD4+ T-cells augmented PAH skin tumorigenesis, CD4+ knockout and wild type mice were compared when subjected to a DMBA skin tumorigenesis protocol. CD4+ knockout mice developed 80% fewer tumors than wild type mice. The percent of mice with tumors and the number of tumors/tumor bearing mice were also decreased in CD4+ knockout mice. These findings are consistent with the hypothesis that, in addition to immune responses against PAH-induced tumors, there are immune responses to the PAHs themselves and that the CD4+ T-cell subpopulation suppresses those host defense mechanisms. Immunopreventive strategies in which the action of CD4+ T-cells is inhibited in individuals predisposed to the development of chemically induced skin tumors may be successful in reducing PAH cancer development.

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Treatment of Murine BMDC with α-MSH Results in the Generation of T-Suppressor Cells

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Intravenous injection of the immunomodulating peptide α-MSH induces hapten-specific tolerance. To investigate the underlying mechanisms, bone marrow derived dendritic cells (BMDC) were generated from Balb/C mice with GMCSF and IL-4. On day 6, cells were treated 3 h with α-MSH (10^{-11} M) and/or DNBS (1 mM) for 2 h. After washing BMDC (5×10^5) were injected i.v. into naive mice. 5 d later, mice were challenged with DNFB and the ear swelling response was measured. To evaluate tolerance induction, mice were resensitized after 2 weeks and rechallenged 5 d later. Mice treated with DNBS pulsed BMDC developed a significant ear swelling response upon challenge with DNFB which was even enhanced after resensitization. In contrast, mice injected with a-MSH exposed BMDC revealed a significantly reduced ear swelling response. In addition, ear swelling was still suppressed following resensitization, indicating that tolerance had developed. Tolerance was hapten specific, since the same animals could be normally sensitized with the unrelated hapten oxazolone. In order to test whether α-MSH treated BMDC induce tolerance via the generation of T-suppressor cells, BMDC were treated with DNBS and/or α-MSH and coincubated with T-lymphocytes derived from lymphnodes of DNFB sensitized mice. Treatment (i.v.) of naive mice with these T-lymphocytes resulted in significant inhibition of chs and induced tolerance against DNFB. Since it was recently shown that the T-suppressor mediating suppression in the model of UV-induced tolerance express CTLA-4, T-lymphocytes in this study were tested for CTLA-4 expression. A-MSH-treated bmdc induced the generation of a subpopulation of $CTLA\text{-}4^+$ (= 5%), $CD4^+$ and $CD8^+$ lymphocytes. These data suggest that α-MSH pulsed dc may cause immunosuppression and tolerance induction through the generation of $CTLA\text{-}4^+$ T-suppressor lymphocytes.

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IL-13 is Required Independently of IL-4 for Epicutaneously Induced Th2 Responses to Soluble Protein

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Unlike contact-induced responses to low molecular weight haptens (e.g. contact dermatitis), epicutaneous (e.c.) exposure to protein antigens can lead to vigorous Th2 type immune responses (e.g. contact urticaria or latex hypersensitivity). We have shown that e.c. sensitization of mice to ovalbumin (OVA) leads to systemic Th2 activation, as evidenced by: (1) OVA-specific IgG1 and IgE responses; (2) lung inflammatory responses following airway challenge with high numbers of eosinophils; and (3) Th2 cytokine production by inflammatory cells (IL-5, IL-13). Furthermore, these Th2 responses are still generated in IL-4 deficient (–/–) mice, but not when IL-4–/– mice are also depleted of IL-13. It was not clear, however, whether IL-13 was simply able to replace IL-4, consistent with previously described redundant roles for these cytokines, or if IL-13 had an independent role in Th2 activation. To address this, IL-13–/– mice were exposed to e.c. OVA (100 μg) on day 0 and then challenged with inhaled OVA (25 μg) from day 14–19. Th2 responses were markedly impaired in IL-13–/– mice, compared to wildtype (WT), in that: (1) OVA-specific IgG1 levels were decreased (53 ± 16 vs. 186 ± 52 μg per ml; $p = 0.02$); (2) serum IgE levels were decreased (130 ± 30 vs. 1603 ± 673 ng per ml; $p = 0.03$); and (3) both the total number of cells recovered by bronchoalveolar lavage (BAL) (2.1 ± 0.2 vs. $15.7 \pm 4.7 \times 10^5$; $p = 0.005$), as well as the number of eosinophils in BAL (0.7 ± 0.2 vs. $132 \pm 43 \times 10^4$; $p = 0.003$) were decreased. In addition, skin-draining lymph node (LN) cells from IL-13–/– mice, isolated 4 days after e.c. OVA exposure and restimulated *in vitro* with OVA, produced reduced amounts of the Th2 cytokine IL-5 (124 ± 29 vs. 591 ± 299 pg per ml), but equivalent amounts of the Th1 type cytokine IFN-γ (9.0 ± 3.8 vs. 10.3 ± 2.9 ng per ml) as compared to LN cells from WT mice. In contrast, induction of contact hypersensitivity to the hapten DNFB, a response mediated by Th1 CD4 and CD8 T cells, was not impaired in IL-13–/– mice. Thus, IL-13 plays a critical role in generation of Th2 responses specifically to epicutaneously encountered antigens.

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Potential Mechanism of CD8 Effector T Cell Dominance in Murine Contact Hypersensitivity to Trinitrophenol (TNP)

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For a variety of allergic reactions to haptens it has recently been shown that CD8+ T cells rather than CD4+ T cells are the crucial effector cells. We have used the murine model of contact hypersensitivity (CHS) to the hapten trinitrophenol (TNP) to analyse the mechanisms underlying the predominance of CD8+ T cells as effectors in this type IV allergic reaction. When mice were immunized by i.d. injection of TNP-modified DC an efficient ear swelling response was detected after ear challenge. The effector cells isolated from regional draining lymph nodes turned out to be cytotoxic, IFN-γ producing Tc1 type CD8+ T cells. Functional as well as phenotypic analysis of the activated effector cells revealed a lack of CD4+ T cell priming despite the presence of immunogenic TNP-epitopes for CD4+ T cells on the antigen presenting cells (APC). In an *in vitro* T cell priming system using DC as APC and T cells and APC from Perforin-deficient and Fas/FasL-deficient mice, we could show that soluble factors do not play a role in the preferential CD8+ T cell priming but that the CD8+ Tc1 effectors lyse the CD4+ T cells in a Fas dependent manner. These findings suggest that bystander cytolysis of CD4+ T cells could be the mechanism underlying the preferential CD8+ T cell activation observed for a number of allergic responses to haptens. This work was supported by the Klinische Forschergruppe "Pathomechanismen der allergischen Entzündung" BMBF FKZ: 01GC9701/7.

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Migratory Properties of Naive, Effector, and Central Memory CD8+ T Cells

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It was recently proposed that different subsets of antigen-experienced T cells may exist that are distinguishable by their preferential ability to home to lymphoid tissues (central memory cells) and nonlymphoid sites (effector cells and effector memory cells). We have shown that antigen primed CD8+ T cells, when cultured in IL-15, resemble central memory cells by phenotype and function ($CD8^{cm}$); $CD8^{cm}$ cells are $CD44^{hi}$, L-$selectin^{hi}$, express CCR7, and mount rapid antigen specific recall responses *in vivo*. In contrast, antigen primed CD8+ T cells cultured in IL-2 ($CD8^{eff}$) are $CD44^{hi}$, L-$selectin^{lo}$, CCR7-, and display effector functions, such as lysis of antigen presenting cells. Here we report the *in vivo* trafficking behavior of $CD8^{cm}$ and $CD8^{eff}$ cells. Both $CD8^{cm}$ and, to a lesser degree, $CD8^{eff}$ cells localized to the T cell area in the spleen, but only $CD8^{cm}$ and not $CD8^{eff}$ cells homed efficiently to peripheral (PLN) and mesenteric lymph nodes (MLN) or Peyer's patches. Intravital microscopy of PLN revealed that $CD8^{cm}$ cells, but not $CD8^{eff}$ cells, rolled and arrested in high endothelial venules. Migration of $CD8^{cm}$ cells to lymph nodes depended on L-selectin and required chemokines that act on CCR7 (i.e. SLC and/or ELC); $CD8^{cm}$ homing to PLN and MLN of plt/plt mice, which do not express CCR7 agonists in lymphoid organs, was reduced by 80% and 87%, respectively. Both antigen experienced subsets, but not naive cells responded to inflammatory chemokines (RANTES, MCP-1, and IP-10) and accumulated at sites of inflammation. $CD8^{eff}$ cells homed ~12-fold more efficiently to the inflamed peritoneal cavity than $CD8^{cm}$ cells, whereas naive T cells were incapable of entering this site of inflammation. In summary, IL-2 treated antigen primed CD8+ effector T cells cells home efficiently to inflamed tissues, but are excluded from entry into most lymphoid organs. Differentiation of antigen primed CD8+ T cells with IL-15 generates central memory-like cells that enter lymphoid organs via HEV and can also migrate to sites of inflammation.

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Epidermis-Infiltrating Lymphocytes Possess a Repertoire of Homing Molecules and Chemokine Receptors Unique Among Tissue-Infiltrating Lymphocytes

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The hypothesis that peripheral blood memory lymphocytes are subdivided into discrete subsets with distinct tissue tropisms has received much experimental support in the past few years. Specific expression of adhesion molecules and (more recently) chemokine receptors has been linked to these subsets. Within the memory CD4 T cell population, the adhesion molecule CLA and the chemokine receptor CCR4 are associated with the skin-homing subset, while integrin $\alpha4\beta7$ and chemokine receptor CCR9 are associated with the small-intestine-homing subset. Here, to shed light on the mechanisms by which cells enter tissues specifically, we have undertaken to characterize CD4 lymphocytes isolated directly from a wide variety of normal and inflamed tissues. We have developed nonenzymatic methods to isolate cells directly from normal and inflamed epidermis. Using the same methods, we have isolated CD4 lymphocytes from lung, synovial fluid, liver, jejunum, ileum, colon (nonlymphoid tissues) and tonsil (a representative secondary lymphoid tissue); for comparison with those from skin. We have found that cells isolated from skin are dramatically enriched in CLA expression, but depleted in $\alpha4\beta7$ expression. Cells isolated from intestinal sites display a reciprocal pattern: depletion of CLA but enrichment of CCR9. Cells isolated from blood and lymphoid tissues consisted of both naïve and memory phenotypes, whereas nonlymphoid tissues contained only memory cells. Activated cells (as assessed by the activation marker CD69) were found in both lymphoid and nonlymphoid tissues, but not in the blood. Expression of both CXCR3 and CCR5 was found on nearly all lymphocytes from nonlymphoid tissues, suggesting that these chemokine receptors do not play a role in tissue-specific homing. CCR4 was expressed at high levels only by lymphocytes derived from skin. CCR4 (but not CLA) was expressed at low levels within lung and inflamed synovial fluid, but never within intestinal sites. CCR4 responses on cells sorted by CCR4 expression levels (assessed by MAbs) demonstrated that only those cells with the highest levels of CCR4 could respond to CCR4 ligands. Thus, functional CCR4 expression may be more tightly associated with skin homing than originally believed.

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Screening of Th2-Specific Anti-Inflammatory Agents and their Inhibitory Effects on Th2-Dominant Allergic Reaction

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In an attempt to topically normalize Th1/Th2 imbalance involved in several inflammatory cutaneous disorders including atopic dermatitis, it is intriguing to seek Th2-specific anti-inflammatory agents instead of potent but nonspecific anti-inflammatory drugs such as corticosteroids. In this study, inhibitory potential against IL-4 and IL-2 production was assessed in culture following incubation with various samples during *in vitro* elicitation of lymph node cells isolated from Keyhole Limpet Hemocyanin (KLH)-sensitized Balb/c mice. The amounts of the cytokines released into the culture medium were measured by ELISA to evaluate the potential for the Th2-specific inhibition. In the long course of seeking such specific agents, we have recently discovered that *Eucalyptus* extract has a characteristic as a Th2-specific anti-inflammatory agent with IC_{50} for IL-4 and IL-2 at a concentration of 0.1% and 0.5%, respectively (5 fold Th2-specific), in contrast to a low Th2-specificity for hydrocortisone with IC_{50} for both IL-4 and IL-2 at a concentration of 0.03 μg per ml. Topical application of the *Eucalyptus* extract to mouse ear lobe at a concentration of 10% during the elicitation with house dust mite antigen (MA) (Th2-dominant allergic reaction) showed an anti-inflammatory effect as revealed by 40% inhibition of the ear swelling. In an organ culture of the elicited ear lobe, the *Eucalyptus* extract applied significantly suppressed the release of IL-4 into the medium without affecting the release of IFN-?. On subsequent fractionation analysis, the most active component has been identified as an aromatic sesquiterpen alcohol, globulol, although the degree of the Th2-specificity was slightly diminished with IC_{50} for IL-4 and IL-2 at 1 μg per ml and 3 μg per ml, respectively (3 fold Th2-specific), compared with the original extract. Topical application of globulol exerted a similar anti-inflammatory effect on the MA-sensitized DTH model at a much lower concentration (0.1%) than the *Eucalyptus* extract did. Our results indicate that topically applied Th2-specific anti-inflammatory agents may be useful in ameliorating Th2-type inflammatory disorders.

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Hsp70 Induction by Artemia Extract Exhibits an Anti-Inflammatory Effect and Down Regulates IL-1 and IL-8 Synthesis in Human HaCaT Cells

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Heat Shock Proteins 70 (Hsp70) play an important role in cell protection against different forms of stress such as thermal shock and UV irradiation. It has been shown that induction of Hsp70 inhibits IL-1 and other cytokines biosynthesis in human cells and, thereby, serves a protective role by suppressing the proinflammatory response. As our prior studies have demonstrated that human cell treatment with Artemia extract 3% induces Hsp70 in a stress-free manner, we investigated the anti-inflammatory effect of Artemia-induced Hsp70 on IL-1α and IL-8 synthesis in irradiated HaCat cells. ELISA assays were performed in order to determine total IL-1α and IL-8 synthesis after UVB exposure. UVB dose course studies (0, 10, 20, 30, 40 mJ per cm^2), and time course experiments (0, 6, 8, 18, 24 h) were performed. The results showed that the dose of choice of UVB to study IL-1α and IL-8 synthesis was 30 mJ per cm^2, and that maximum synthesis occurred 6–8 h after UVB radiation for IL-1α, 18–24 h for IL-8. Moreover, our studies demonstrated that, depending on UVB doses, IL-1α and IL-8 level of synthesis decreases considerably in the Hsp70-induced cells, by 20–30% and 25–40%, respectively, compared to the control cells. Interestingly, maximum decrease in IL-1α in these cells was observed 18–24 h after UV. Statistical treatment of these results by the Student's *T*-test ($p < 0.05$) showed that the decrease of synthesis level of these two related cytokines is significant. Our studies confirm the anti-inflammatory effect of Hsp70 and propose a very interesting and new approach to inhibiting the inflammatory response via stress-free Hsp70 induction in the cells.

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Artemia Extract Induces Hsp70 in Human Cells and Enhances Cell Protection from Stress

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Heat shock proteins (Hsps) or molecular chaperons, play an important role in protecting cells from different types of stress (in particular Hsp70). In mammalian cells, under nonstressful conditions, Hsps are significant for cell development, differentiation and for protein folding and assembly. As we previously studied the multiple cell-protective effects of Artemia extract, we investigated whether the induction of Hsp70 was one of these protective mechanisms. Different time and dose course studies showed that Artemia extract 3% significantly induced Hsp70 synthesis in cultured human fibroblasts, HaCat and A431 cells. In fibroblasts, Northern blot studies revealed Hsp70 mRNA expression within 3 h of Artemia extract application. The protein profile of Hsp70 shows a moderate increase in the first hour, probably due to the translation of some pre-existent Hsp70 mRNA in the cell, followed by a stronger and consistent increase 3, 6, and 24 h later. Other studies strongly suggest that this induction of Hsp70 is stress-free. As stress is the condition that usually triggers Hsps synthesis, we have the first evidence of a stress-free Hsp induction mechanism. In order to investigate the ability of stress-free Hsp70 induction in enhancing cell physiological protection from stress, we exposed the cells to a thermal shock (47°C for 1 h). Cells treated with Artemia were less affected and more resistant to the heat insult than the untreated cells. Moreover, studies on an *ex vivo* model confirmed these results and showed that Artemia extract 3% application in a cream formula into the skin significantly induced Hsp70 protein in the skin's keratinocytes and fibroblasts, compared to the placebo-treated control. Moreover, Artemia-induced Hsp70 skin samples exposed to heat stress presented a well-preserved structure and minimal heat-injury signs compared to placebo-treated skin. These studies demonstrate that Artemia extract induces Hsp70 in human cells and skin. This induction is stress-free and increases skin defense and resistance to stress.

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Tumor Necrosis Factor Receptor-I Pathway Involved in Arsenic Induced Human T-Helper Cells Apoptosis

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Epidemiological studies demonstrated that long-term exposure to arsenic induces arsenical skin cancers, including Bowen's disease. Patients with Bowen's disease showed a defective immunological function and a decreased T-helper subpopulation in peripheral mononuclear cells (MNC). The purpose of this study was to investigate the effects of arsenic on T cell survival and functions in MNC. The cytotoxicity of arsenic was detected by XTT assay. Cell cycle analysis and TUNEL staining of apoptotic cells were measured by flow cytometry. We also detected the apoptosis associated TNF receptor-I (TNF-RI) expression on T cells using double stained flow cytometric analysis. The apoptosis related protein expression in TNF-RI pathway were detected by Western blotting. Arsenic concentrations higher than 1 μM induced cytotoxic effect on T cells. When exposed to arsenic, MNC cell cycle was arrested at G0/G1 phase, at the same time, apoptotic cell death was induced. CD4+ T-helper cells were the major apoptoic population in MNC. TNF-RI expression on CD4+ cells was significantly enhanced by arsenic treatment when compared to other cells in MNC. This finding may provide a reasonable explanation that T-helper cells are sensitive to arsenic stimulated apoptotic induction. Increased expressions of TNF-RI related proteins, i.e. TNF-RI associated death domain protein, Fas-associating protein with death domain and activated caspases were observed. TNF-RI plays an important role in apoptotic signaling in various physiological and pathological conditions, especially in immunological reactions. Our results indicate that TNF-RI signaling is involved in arsenic induced apoptosis in T-helper cells.

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The Regulatory Roles of Adenylate Cyclase and Cyclic Nucleotide Phosphodiesterases 1 and 4 in Interleukin-13 Secretion by Activated Human T Cells

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We studied the activities of cAMP-synthesizing adenylate cyclase (AC) and cAMP-hydrolyzing cyclic nucleotide phosphodiesterase (PDE) in PHA- or anti-CD3 plus anti-CD28-stimulated human T cells, and examined their roles in IL-13 secretion. PHA- or anti-CD3/CD28-induced IL-13 secretion was blocked by AC inhibitor MDL 12 330 A, PDE 1 inhibitor 8-methoxymethyl-3-isobutyl-1-methylxanthine, or PDE4 inhibitor rolipram. AC in T cells was transiently activated 5 min after stimuli, followed by the transient activation of PDE4 at 30 min PDE1 activity, undetectable in resting T cells, was detected 3 h after stimuli, then gradually increased. Anti-CD3/CD28, but not PHA, increased PDE1, 2, 3, and 4-independent PDE activity, and the increase was blocked by PDE7 antisense oligonucleotide. PHA or anti-CD3/CD28 newly induced PDE1B mRNA expression. Neither stimulus altered PDE4 mRNA level. Anti-CD3/CD28, but not PHA, increased PDE7 mRNA level. The cAMP level of T cells increased 5 min after stimuli, returned to the basal level at 2 h, then continued to decrease. These results suggest that PHA or anti-CD3/CD28 initially (= 5 min) increases cAMP in T cells via AC, then reverses the increase via PDE4 (= 2 h), and in the later phase (2 h) further decreases cAMP via PDE1. Both the time-dependent increase and decrease of cAMP may be required for IL-13 secretion.

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Identification and Functional Characterization of Human CD4+ CD25+ T-Cells with Regulatory Properties Isolated from Peripheral Blood

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CD4+ CD25+ T-cells represent a unique population of regulatory T-cells that suppress the activation of conventional T-cells. So far these cells have only been identified in rodents. This study shows that comparable ''professional'' suppressor T-cells are also present in human blood. Human CD4+ CD25+ or CD4+ CD25- T-cells were sequentially enriched by immunomagnetic beads from peripheral blood. A subpopulation of human CD4+ CD25+ T-cells (but not CD25- T-cells) express CD45RO, HLA-DR and intracellular CTLA-4, do not expand following stimulation with mature allogeneic DC, PHA, or anti-CD3/CD28. Furthermore, CD4+ CD25+ T-cells markedly suppress the expansion and cytokine production of conventional CD4+ CD25- or CD8+ T-cells in a cell contact and activation dependent manner. CD4+ CD25+ T-cells express CTLA-4 on the membrane after activation, which is detectable for several weeks thereafter. The anergic state of CD4+ CD25+ T-cells was not reversed by addition of anti-CTLA-4 or anti-TGF-β or anti-IL-10 antibodies. Transwell experiments demonstrated that the suppressive properties of human CD4+ CD25+ T-cells are dependent on direct cell contacts between regulatory and conventional T-cells. Moreover, regulatory T-cells showed a cell cycle arrest in the G1/G0 phase and no detectable production of IL-2, IL-4, or IFN-g either on the protein or mRNA level. The anergic state of CD4+ CD25+ T-cells was partially reversible by addition of IL-2 or IL-4. These data demonstrate that human blood contains a resident T cell population with potent regulatory properties.

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IL-4 Reverts DNA-Induced Th1-Development *In Vitro* and *In Vivo*

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Bacterial DNA has been shown to induce IFN-gamma producing CD4+ T cells (Th1) *in vitro* and *in vivo*. This effect can be mimicked by single stranded oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN). Most strikingly, CpG-ODN are capable of redirecting established IL-4 mediated Th2 immune responses into protective Th1 responses in murine models of leishmaniasis and asthma. In order to study the interaction of CpG-ODN and IL-4 during T cell differentiation, we stimulated naive transgenic CD4+ T cells from DO11.10 mice *in vitro* with the specific peptide (OVA) and antigen presenting cells (APC) in the presence of either CpG-ODN or CpG-ODN + IL-4. Priming of Th cells in the presence of CpG-ODN induced Th1-differentiation as T cells secreted high amounts of IFN-gamma but no IL-4 upon restimulation. In contrast to data obtained with L. major infection or asthma, addition of IL-4 during T cell priming completely inhibited CpG-ODN mediated Th1-induction. Instead, T cells developed a Th2 phenotype marked by strong antigen-specific IL-4- and suppressed IFN-gamma-production. In order to evaluate the significance of our findings for the *in vivo* situation, we transferred OVA-specific CD4+ T cells into BALB/c mice and immunized the mice with ovalbumin in the presence of either CpG-ODN or CpG-ODN + IL-4. CD4+ T cells isolated from mice immunized in the presence of CpG-ODN developed a Th1 phenotype and, again, IL-4 inhibited CpG-ODN induced Th1-induction as Th cells isolated from mice immunized in the presence of CpG-ODN + IL-4 showed an IL4+/IFN-γ- Th2-phenotype. Since IL-4 is capable of suppressing CpG-ODN induced IL-1, IL-12(p35) and IL-12(p40) mRNA-expression of SEB-stimulated spleen cells, IL-4 may inhibit CpG-ODN induced Th1-differentation through a direct effect on freshly activated APC.

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***In Vitro* Generation of ''Central Memory'' T Cells**

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Administration of *in vitro* activated antigen-specific autologous T cells is becoming an important approach in cancer immunotherapy. By virtue of their having been exposed to antigen, these T cells bear memory T cell markers, such as CD45RO. Very recently memory T cells have been subclassified into two groups based upon their homing properties. (1) ''Effector memory'' T cells bear tissue-specific homing markers (CLA, α4β7) and cannot enter lymph nodes from blood due to their lack of expression of CCR7 and L-selectin. (2) In contrast, ''central memory'' T cells are defined by their expression of L-selectin, CCR7 and LFA-1, molecules absolutely required to enter lymph nodes from blood. ''Central memory'' T cells are thought to confer long-term memory. We reasoned that effective, lasting immunotherapy would require the transfer of central memory T cells. As a first step to approach this problem, we cultured T cells under conventional activating conditions with anti-CD3 and IL-2. T cells cultured under these conditions expressed low levels of L-selectin or CCR7 but virtually no cells expressed both markers. Thus, T cells cultured under conventional conditions were almost exclusively effector memory T cells. In contrast, memory T cells cultured in the presence of IL-7 coexpressed high levels of the molecules that define central memory T cells: L-selectin and CCR7. It may be that the efficacy of autologous T cell therapy can be enhanced by optimizing conditions that favor central memory cell generation *in vitro*.

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Differential Regulation of CTLA-4 in Human Memory and Naive CD4+ T Cells

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Immunologic memory is a cardinal aspect of the immune response and is acquired upon exposure to antigen. Proper development of memory is essential for elimination of antigen. Abnormalities in memory T cell function can lead to diverse cutaneous and systemic inflammatory diseases. At the molecular level, the basis of T cell memory remains poorly characterized, particularly in the CD4+ subset. It is believed that memory cells undergo biochemical and genetic changes that permit these cells to respond qualitatively and quantitatively more robustly to an antigenic stimulus. One surface marker, CD45RO, has been used to separate memory CD4+ T cells from naïve T cells. To study the molecular differences between these two subpopulations, we isolated genes that were expressed preferentially in CD45RO+,CD4+ T cells by a PCR based, suppressive-subtraction approach. Numerous genes were identified that were expressed at a greater level in memory T cells. One gene identified by this method and is important in immune function is CTLA-4 (CD152). CTLA-4 shares homology to CD28 and binds costimulatory molecules B7-1 and B7-2 to regulate T cell activation. By RT-PCR, the level of CTLA-4 expression was greater in CD45RO + T cells by 7 fold. In addition, the protein level for CTLA-4 as measured by immunoblots was higher in the CD45RO+ population. Upon activation of memory T cells, CTLA-4 was induced with a more rapid kinetics in comparison to naïve T cells at the message level and the protein level. These findings indicate that the mechanism regulating CTLA-4 expression differs in naïve and memory T cells, both in the resting and activated state. These findings suggest that studying the regulation of CTLA-4 expression in naïve and memory T cells may lead to an understanding of memory T cell development and insight into mechanisms of memory T cell-mediated diseases.

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A Critical Role for CCR6 in the Arrest of CLA^+ Memory T Cells on Microvascular Endothelia Under Physiologic Flow Conditions *In Vitro*

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CC chemokine receptors 4 and 6 (CCR4 and CCR6) are expressed by skin-homing memory T cells (mTC) in psoriasis and may be involved in T cell recruitment to skin. We asked whether one or the other was required for firm arrest of mTC on activated human dermal microvascular endothelial cells (HDMEC) *in vitro*. By real-time quantitative RT-PCR, LARC (a CCR6 ligand) mRNA was up-regulated 170-fold in TNF-α-stimulated HDMEC whereas TARC (a CCR4 ligand) was minimally expressed. $CD45RA^-$ human mTC (15–40% CLA^+) enriched from peripheral blood were injected into a parallel plate flow chamber at 1.5 dynes per cm^2 using either an activated HDMEC monolayer or recombinant chemokines and adhesion molecules coated onto plastic as substrates for rolling and arrest. T cell arrest after 5 min of flow was quantified with motion analysis software. LARC (vs. no chemokine) increased arrest of CLA^+ mTC as well as of CCR6-transduced Jurkat T cells by 6-fold in flow assays with recombinant E-selectin/ICAM-1. Rolling and arrest of mTC on activated HDMEC was E-selectin- and CLA-dependent. Exposure of mTC to receptor desensitizing levels (1 μg per ml) of LARC caused a 50–75% decrease ($n = 8$, $p < 0.001$) in arrest of mTC that was indistinguishable from the decrease observed when mTC were treated with pertussis toxin ($n = 8$, $p > 0.5$), which inactivates Gi-protein-coupled chemokine receptors. By contrast, exposure of mTC to TARC and SLC (a CCR7 ligand) had no effect on arrest. Furthermore, mTC depleted of $CCR6^+$ cells by magnetic beads arrested poorly on HDMEC. By confocal microscopy, CCR6 redistributed to the leading edge of polarized mTC after arrest on HDMEC. Our results clearly demonstrate that CCR6 expression by skin-homing mTC is a critical component in chemokine receptor-mediated arrest of mTC *in vitro* and is likely to contribute to the recruitment of mTC to inflamed skin *in vivo*.

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Very Late Antigen (VLA)-5 Costimulation of CD4+ T Cells: Relevance to UV-Induced Immunotolerance

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Macrophages that infiltrate UV-exposed skin (UV-Mph) are associated with the induction of antigen-specific tolerance. We previously showed, in humans, that the activation of CD4+ T cells (TC) by UV-Mph differs from that by Langerhans cells (LC); including deficient expression of IL-2 receptora-chain (CD25), induction of type 2 cytokines, and distinct costimulatory molecule and growth factor use by UV Mph-, relative to LC-activated TC. We now explore whether integrin signaling directs distinct TC activation by UV-Mph. Blocking TC VLA-5 a-chain (CD49e) with antibodies (Ab) inhibited UV-Mph, but not LC-stimulated growth in mixed epidermal cell-lymphocyte reactions (to 50 ± 23% vs. 110 ± 15% of control Ab-containing cultures). We then explored the role of CD49e in TC activation using these Ab. Activation through CD49e and CD3 increased TC growth 180 ± 17% relative to through CD3 alone. Using immunoprecipitation and Western blotting, alterations in protein phosphotyrosine patterns are seen upon addition of anti-CD49e to anti-CD3 stimulation including increased FAK and jun and reduced src. Although augmented by stimulation through CD49e and CD3, FAK phosphorylation was markedly reduced and Pyk2 was elevated by the addition of CD28 stimulating Ab. Flow cytometry showed CD3 stimulation for 24 h caused 17 ± 9% of TC to become CD25+ 0. Addition of anti-CD28 Ab increased this to 29 ± 11%. In contrast, only 9 ± 3% of CD3/CD49e-stimulated TC up-regulated CD25. Interestingly, simultaneous stimulation through CD3, CD28 and CD49e up-regulated CD25 on only 13 ± 7% TC. These results demonstrate that a5 integrin (CD49e): (1) is used during TC activation by UV-Mph, but not by LC (2) provides true costimulation rather than merely adhesion, and (3) induces CD25-deficient TC activation as do UV-Mph. Also, these results suggest that depending on context, a5 integrin signaling may have a greater role in adhesion (via FAK signaling) or TC activation (via src or Pyk2 signaling). We conclude that differences in immune outcome mediated by UV-Mph (tolerance) and LC (sensitization) are brought about in part by differences in costimulatory signals provided to TC and that a5 integrin signaling is important to TC activation by UV-Mph but not LC.

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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.