Instituto Juan March
de Estudios e Investigaciones

55 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Biology of Skin and Skin Diseases

Organized by

D. R. Roop and J. L. Jorcano

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INTRODUCTION

D.R. Roop and J.L. Jorcano

The skin is the largest organ of the human body. Its importance is well known as a protective barrier against the aggression of many external agents, physical, chemical and biological (bacteria, fungus, virus, etc.). Nonetheless, attention to and research on this tissue have long been scarce, even though dermatological diseases affect a large number of patients (for instance, psoriasis, chronic ulcers and cutaneous tumors are high-incidence diseases in industrialized countries).

In recent years, this situation has changed. Recognition that the epidermis forms a complex, interesting and highly-ordered system in which the processes of proliferation, differentiation and cell death can be studied has attracted the attention of a growing number of investigators. In a spectacular race against the clock, the molecular basis of several skin diseases has been described and significant progress has been made in understanding the mechanisms controlling proliferation and differentiation in this tissue, as well as in the identification of the factors that intervene in these processes. Due to the barrier function of the skin, special attention should be given to the advances in cytoskeletal organization and its role in the maintenance of epithelial structure and function, and in particular to the keratins and to the molecules and structures of cell adhesion (desmosomes and hemidesmosomes, integrins, cadherins, etc.).

The external corporal localization of the skin and the identification of those sequences that direct gene activity in this tissue have made the use of transgenic animals a flexible and widely-used technique which permits the acquisition of important information *in vivo*. The skin has thus become one of the tissues on which more research is being done and more progress is being made.

From the clinical point of view, three novel characteristics have attracted considerable attention to this tissue:

1) The finding that the skin produces interleukins and other cytokines of enormous importance in the infectious and inflammatory processes of this tissue. Given the large area of the skin, it is hypothesized that these epidermal cytokines, after passing to the circulation, may also play a very important systemic role.

2) At present, the skin is the organ with the highest incidence of tumors, and this frequency continues to increase. There is evidence that many of these tumors are caused by increased exposure of the skin to ultraviolet radiation, due as much to the deteriorating ozone layer as to reigning esthetic fashions. Skin carcinogenesis is an area in which considerable work is being done.

3) *In vitro* culture and expansion of human keratinocytes are recently-developed methods of great relevance, both in patients with major cutaneous lesions (such as, for example, those with burns covering a large body area, insuperable until this new technique was introduced) and in the treatment of ulcers which do not respond to classical therapies. In addition, however, accumulated experience and the relative ease of *in vitro* culture of epidermal keratinocytes and their subsequent transplant in patients make the epidermis an ideal tissue for the development of gene therapy protocols, for both hereditary and tumoral diseases. Recent advances in the identification and isolation of epidermal stem cells are particularly relevant in this context, as is the development of methods which permit the stable or transitory expression of therapeutic genes in keratinocytes.

These subjects were addressed in depth and actively discussed in the present Workshop.

Epidermal stem cells

Chairperson: Stuart H. Yuspa

Clonal analysis of multiplying keratinocytes

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The epidermis is a self-renewing pluristratified epithelium of which keratinocytes are the predominant cell type. Keratinocytes are either proliferative or post-mitotic differentiated cells. Those keratinocytes which have the capability to multiply are either stem cells with an extensive growth capacity, or transit amplifying cells of restricted growth capacity.

Some multiplying keratinocytes have the ability to initiate colonies *in vitro*. These clonogenic keratinocytes, which are also termed keratinocyte colony-forming cells (K-CFCs), have different growth capacities (Barrandon and Green, 1987). The holoclones have the most growth potential and are stem cells (Rochat et al., 1994). They generate meroclones which have also a significant growth potential. However meroclones generate, with an increasing frequency when serially passaged, colony-forming cells which growth capacity is restricted to a maximum of 15 divisions (from one to fifteen). These paraclone-forming cells are thus transit amplifying cells (Barrandon and Green, 1987; Jones and Watt, 1993). The conversion from holoclone to meroclone and then to paraclone is a unidirectional and irreversible process which molecular mechanisms are still unknown. However, inappropriate culture conditions, serial culture passages as well as natural aging, are known to affect clonal conversion. It should be again emphazised that adequate culture conditions are indispensable when human K-CFCs are used to generate cultured epithelium for clinical applications (treatment of extensive burn wounds, gene therapy). It should also be emphasized that there is usually enough stem cells in a small skin biopsy, even in elderly patients, to generate large quantities of cultured epithelium. Accordingly, one of the most important question is then: how stem cells should be manipulated so that there stemness is protected when they are cultivated *in vitro* and transplanted *in vivo*?

The ability to identify and to localize stem cells precisely within the epidermis and its appendages, is of great importance. However, a keratinocyte stem cell can solely be recognized by its function, i.e. its ability to initiate a progressively growing colony in culture and to generate a functional epithelium when transplanted (Barrandon and Green, 1987; Jones and Watt, 1993; Rochat et al., 1994). Clonal analysis have demonstrated that the K-CFCs described *in vitro* are also inhabitants of human epidermis and hair follicles. However, the small number of paraclones that is obtained from freshly dissociated epidermis or hair follicles, strongly suggests the existence of a second population of transit amplifying cells *in vivo* (Barrandon, 1993). These multiplying cells, which cannot initiate colonies under current culture conditions, are likely to be those identified by cell kinetics experiments *in vivo* (Potten,1983; Lavker and Sun, 1982; our results). Therefore, the exact role of paraclones

during normal epidermal renewal is unclear, but it can be that of an emergency cell population which is recruited when a large pool of multiplying cells is needed, e.g. during wound healing.

In mouse pelage hairs, follicular stem cells are thought to be located in the bulge, which is the site of insertion of the arrector pili muscle and appears as a small protuberance of the outer epithelial sheath (Cotsarelis et al., 1990). However, it is clear that the location of follicular stem cells differ among species and among hair follicle types. For instance, most keratinocyte colony-forming cells are located in the upper part of the rat vibrissa in a bulged area which is not the insertion of the arrector pili muscle (Kobayashi et al, 1993). Similarly, most stem cells of a human hair follicle are segregated in a part of the outer epithelial sheath which is distant from both the site of insertion of the arrector pili muscle and the hair bulb (Rochat et al., 1994). Most importantly, stem cell segregation appears to be a characteristic of all hair follicles as it is also found in the mouse vibrissa as well as in rabbit pelage hairs (Schmitt et al., 1996). Although the physiological significance of this segregation remains unclear, it is likely to serve as a reservoir from which stem cells can be recruited to maintain hair growth or to heal a cutaneous wound. The later implies that some follicular stem cells are bipotent, i.e. that they have the capability to generate two different epithelial cell lineages, that of the hair follicle and that of the epidermis.

One of the most striking features of a hair is its carefully regulated growth cycle. Although the cellular and molecular mechanisms that occur during hair cycle, are not fully understood, the presence of stem cells is necessary to maintain hair growth for a long period of time. We have examined the influence of the hair cycle on the distribution of K-CFCs in rat vibrissal follicles. Important modifications in the distribution of K-CFCs occured when a new growth cycle was initiated. Surprisingly, the number of KCFCs increased in the part of the follicle which underwent the most important modifications during catagen phase. This pattern was highly reproducible. A normal distribution of the K-CFCs was then resumed as the growing phase (anagen) proceeded. Furthermore, BrdU labelled cells were also present in this part of the follicle indicating that K-CFCs were actively multiplying. It is worth noting that the number of BrdU labelled cells located in the bulge of the vibrissal follicle did not increased significantly when a new growth cycle was initiated. Our results provide the first evidence that there are important modifications in the distribution of K-CFCs in the vibrissal follicle when a new growth cycle is initiated.

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Keratinocyte Stem Cells: Their Location and Mesenchymal Messages

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Keratinocyte stem cells have been identified in the limbal region of corneal epithelium, the bottom of deep rete ridges of palm/sole epithelium, and more recently in the outer root sheath of the upper follicle. The stem cells from these diverse tissues share certain features including a slow-cycling kinetics, a primitive cytology, and a unique environmental niche (Cell 57:201, 1989; Seminar Develop. Biol. 4:405, 1993). In the case of the hair follicle, recent studies from tritium-labeling and cell culture studies suggest that follicular stem cells are not located in the bulb region, as was previously thought, but rather reside in the upper follicle in an area near the bulge (Cell 61:1329, 1990). Interactions between the dermal papilla cells, a group of specialized mesenchymal cells of the follicle, and the normally slow-cycling epithelial stem cells may play a crucial role in initiating the next anagen. To better understand the molecular basis of this mesenchymal/epithelial interaction, we have screened for genes that are expressed specifically in the dermal papilla cells but not in the closely related fibroblasts using the random primed-PCR technique. A number of genes have been identified to fit this criterion. One of them encodes nexin 1, a potent serine protease inhibitor. The mRNA of nexin 1 was found to be dermal papilla-specific and underwent hair cycle-dependent changes reaching a peak level during anagen. The level of nexin 1 mRNA in several permanent rat vibrissa papillary cell lines varied significantly, and these levels correlated well with the cell lines' ability to support in vivo hair follicular reconstitution. These results suggest that nexin 1 may play a role in regulating follicular growth and differentiation (J. Cell Sci., in press). Studies of these genes which encode secretory products that can modulate proteases and extracellular matrix components may lead to a better understanding of follicular regulation.

ROLE OF CELL ADHESION MOLECULES IN REGULATING KERATINOCYTE GROWTH AND DIFFERENTIATION

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Within the epidermis the basal layer of keratinocytes adheres to the underlying basement membrane via receptors of the integrin family. In all of the living epidermal layers intercellular adhesion is dependent on cadherin receptors, the classical cadherins (E- and P-cadherin) and the desmosomal cadherins (desmocollins and desmogleins). My laboratory is using a combination of cell culture experiments and studies in transgenic mice to study the functions of integrins and classical cadherins in regulating keratinocyte growth and differentiation.

We have found that integrins not only mediate adhesion of keratinocytes to extracellular matrix proteins, but also ensure that committed cells are selectively expelled from the basal layer. Ligand binding by the β_1 integrins serves as a negative regulator of terminal differentiation. The keratinocytes with the highest proliferative potential, stem cells, express higher integrin levels than other cells within the basal layer. Loss or misexpression of integrins profoundly affects cell behaviour.

E- and P-cadherin are required for assembly of keratinocytes into stratified, or multilayered, sheets. When cadherin function is inhibited proliferation is reduced and the proportion of cells that undergo terminal differentiation is increased. Epidermal stem cells express lower levels of E-cadherin than other basal cells.

The conclusion from our studies is that cadherins and integrins play important and complementary roles in regulating the growth and differentiation of keratinocytes. There are some data to suggest that there is cross-talk between the different classes of receptor and the mechanism is currently under investigation.

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CLONOGENIC KERATINOCYTES IN EPIDERMAL HYPERPLASIA AND CARCINOGENESIS IN THE MOUSE.

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Regenerative epidermal hyperplastic growth is both necessary and sufficient for the promotion of cutaneous neoplasms following a subtumorigenic exposure to a carcinogen, but its cellular mechanism remains unknown. A single application of 17 nmols of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to CD-1 female mice induces within 1 day a 2-fold increase in epidermal thickness that persists until day 4, after which the epidermis returns to normal thickness. Twice weekly applications of TPA to uninitiated skin maintain the epidermal thickness. Twice weekly applications of TPA to initiated epidermis promote early papillomas by 6 weeks. To explore the cellular mechanism of hyperplastic growth, we investigated the role of the keratinocyte colony forming units (kCFU) with high proliferative potential in the production and regression of epidermal hyperplasia induced by single and multiple applications of TPA to control mice and to mice initiated with 200 nmols of dimethylbenz[*a*]anthracene. We harvested epidermal cells from groups of mice at intervals following treatment, seeded them at clonal density onto feeder layers, and cultured them for 2 to 4 weeks. Surprisingly, one day following a single application of TPA to uninitiated skin when the epidermis was increasing in thickness and cell number, the number of kCFU did not increase over acetone controls. Likewise, 4 to 12 applications of TPA to uninitiated skin failed to increase the number of kCFU. However, the number of kCFU increased 3-fold over control at 6 days following treatment with TPA, when the epidermis was regressing to normal thickness. Multiple applications of TPA to initiated epidermis increased both the size and number of kCFU in a time-dependent manner. These results suggest a tight control on the number of kCFU during epidermal hyperplasia, but a deregulation during the two-stage carcinogenesis.

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Epidermal growth and differentiation

Chairperson: José Luis Jorcano

THE ROLE OF THE RETINOID SIGNALLING PATHWAY IN FORMATION AND MAINTENANCE OF EPIDERMAL BARRIER FUNCTION. D.R. Roop[1], J.R. Bickenbach[1], P.S. Attar[1], S. Imakado[1], J.A. Rothnagel[1], P.W. Wertz[2]. [1]Baylor College of Medicine, Houston, Texas, [2]The University of Iowa, Iowa City, Iowa.

Previous transgenic mice resulting from targeted disruption of retinoic acid receptors (RARα or γ) or retinoid X receptor (RXRα) genes either failed to exhibit a skin phenotype, suggesting functional redundancy among RARs, or died *in utero* prior to formation of a functional epidermis. To produce a functional knock-out of the retinoid signalling pathway with a single targeting event, we expressed a mutant RARα (RARα403), which exhibits a broad spectrum of potent dominant negative activity due to its ability to form inactive heterodimers. To avoid embryonic lethality, we targeted expression of RARα403 with an epidermal-specific vector which is expressed late in mouse development. This approach produced a profound skin phenotype resulting in neonatal lethality within 24 hours. Assays for transepidermal water loss demonstrated that phenotypic skins lost water three times faster than controls and electron microscopy revealed the absence of lipid lamellar structures in the stratum corneum of phenotypic epidermis, suggesting a defect in lipid metabolism. These transgenic results, implicating the retinoid signalling pathway in modulation of lipid metabolism are novel for the epidermis, but not for other tissues. In the liver, RXRs form heterodimers with peroxisome proliferator activated receptors (PPARs), which are also members of the nuclear hormone receptor superfamily and regulate expression of genes encoding hydrolytic enzymes. To examine the potential role of this pathway in generating the phenotypes observed in our transgenic mice, we have determined that PPARs are expressed in the epidermis. Furthermore, we have demonstrated that transfection of RARα403 into primary keratinocytes blocks transactivation of a PPAR reporter construct, probably due to the ability of RARα403 to bind to and sequester RXRs. Hydrolytic enzymes are required for formation of multilamellar lipid structures between corneocytes. An analysis of the lipid composition of phenotypic epidermis has confirmed a defect in lipid processing. Our results suggest that genes which encode enzymes involved in hydrolysing epidermal lipids may be regulated by RXRs/PPARs and their cognate ligands.

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REGULATION OF EPIDERMAL DIFFERENTIATION BY THE PROTEIN KINASE C PATHWAY. Stuart H. Yuspa, Andrzej A. Dlugosz, Mitchell F. Denning, Yun Sil Lee and Susan Rutberg, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD. USA

Mouse keratinocytes express 5 isoforms of protein kinase C ($\alpha,\delta,\epsilon,\zeta,\eta$ (1). During Ca^{2+}-induced keratinocytes differentiation, intracellular diacylglycerol levels increase and protein kinase C (PKC) α,δ and ϵ translocate from the cytosol to the particulate fraction indicating enzyme activation (2). Pharmacological activation of PKC by treatment of cultured keratinocytes with phorbol esters or exogenous diacylglycerol induces transcription of late markers of keratinocyte differentiation such as loricrin, filaggrin, sprl and keratinocyte transglutaminase, accelerates cornification, and inhibits expression of keratins 1 and 10 (3,4). Pharmacological inhibitors of PKC prevent late marker expression and block cornification (3,4). Together , these results indicate protein kinase C is an important signal transducing pathway that regulates the terminal phase of keratinocyte differentiation. Among the isoforms of PKC involved in keratinocyte differentiation, PKC α seems particularly relevant to expression of differentiation markers, since inhibition of this isoform by specific antisense oligonucleotides reduces the expression of loricrin , filaggrin and keratinocyte transglutaminase. Furthermore, PKC δ may also be relevant to terminal differentiation since this enzyme is tyrosine phosphorylated late in the terminal phase of maturation. To determine the nuclear target through which PKC activation controls transcription of keratinocyte-specific gene expression, the AP-1 family of transcription factors was analyzed in cultured keratinocytes induced to differentiate by Ca^{2+}. DNA binding activity of Fra1, Fra 2, c-Jun, Jun B and Jun D increased in differentiating keratinocytes. Increased DNA binding was associated with an increase in the nuclear content of these transcription factors and in some cases to their modification by phosphorylation (5) and involved activation of PKC, particularly PKCα (5). Paradoxically, overall levels of AP-1-mediated transcriptional activity were decreased in differentiating keratinocyte suggesting a role for AP-1 as a negative regulator of gene expression. While c-Fos was not a component of the AP-1 DNA binding complex during normal keratinocyte differentiation, elevation of c-Fos by pharmacological induction or by transfection of a c-*fos* expression plasmid changed the AP-1 activity from a transcriptional repressor to a transcriptional activator. These results suggest that the activity of PKC and AP-1 in regulating keratinocyte gene expression is determined by the complement of AP-1 family members expressed at the time keratinocytes receive the differentiation signal. Factors or conditions that alter the activation state of keratinocytes will modify the expression of keratinocyte-specific differentiation markers in response to a signal to differentiate. These results may explain ambiguities in the interpretation of expression data in studies using keratinocytes cultured under diverse conditions and reveal a mechanism whereby the program of keratinocyte differentiation is altered in pathological syndromes.

Targeting stromal proteinases in the treatment of epithelial malignancies.

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The matrix metalloproteinases (MMPs), also known as matrixins (1), are extracellular zinc-enzymes that mediate a number of tissue remodeling processes, including those associated with cancer progression (2). Most MMPs have been shown to cleave at least one component of the extracellular matrix (ECM) (3). This observation has led to the concept that during tumor progression cancer cells express MMPs in order to disrupt the ECM and invade adjacent tissues (4). However, it has been found that some MMPs implicated in the progression of human carcinomas were not expressed by the cancer cells themselves, but by stromal cells surrounding them. Thus, stromelysin-3 (ST3), gelatinase A, and more recently MT-MMP1 have been found to be specifically expressed in fibroblastic cells of human carcinomas (5 and refs therein).

The stromal expression of MT-MMP1 in human carcinomas is of particular importance. This membrane-bound MMP is a progelatinase A activator (6). Gelatinase A is secreted as a zymogen (progelatinase A) which is believed to be activated at the membrane cell-surface by binding directly or indirectly to MT-MMP1 (7, 8). The observation that progelatinase A and its membrane-bound activator are expressed by the same fibroblastic cells in human carcinomas suggests that stromal cells are both an important source of proteolytic activities and a target for at least part of these activities. This finding also suggests that during human carcinoma progression MMPs contribute to aspects of the malignant phenotype other than the direct promotion of cancer cell invasion. This possibility is also consistent with observations showing that MMP expression in stromal cells of human carcinomas can be detected before they become invasive (9).

Synthetic MMP inhibitors are believed to represent a new class of potential anticancer agents (10). Targeting proteinases produced by stromal cells rather than cancer cells may have several advantages, and would allow the identification of new antitumor agents capable of acting synergistically with those presently used. Although broad spectrum inhibitors have been successfully developed (11), it is currently believed that inhibitors specific for each MMP should be designed (10). In this context, ST3 which has been shown to increase the tumor take of human cancer cells in nude mice (12), is an attractive target. ST3 has been found to be over-expressed in most human invasive carcinomas (9), and it exhibits unusual functional properties. In contrast to other MMPs, ST3 cannot cleave any of the major ECM molecules (13, 14), and the proST3 activation pathway is so far unique among MMPs (15, 16).

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PREVENTION OF SCARRING DURING ADULT WOUND HEALING

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Scarring following wound healing is a major medical problem with adverse aesthetic functional and growth sequelae. Control of scarring is of major importance following surgery or trauma to any body organ.

Wounds made on mammalian embryos during the first half of incubation heal perfectly with no evident signs of scarring. We have investigated the cellular and molecular basis of this scar free embryonic healing. A number of experiments indicate that scar free healing is not related to the unique warm, moist, sterile, embryonic environment. A major difference between embryonic wounds which heal without scarring and adult wounds which scar is the inflammatory cell infiltrate and hence growth factor profile at the wound site. In the embryo the immune system is poorly developed. Consequently, there is a markedly reduced and qualitatively different inflammatory cell infiltrate. Levels of growth factors, such as TGFβ1 and 2, are markedly reduced compared to adult wound healing. We have utilized this information to experimentally manipulate adult wounds. Exogenous topical application of neutralising antibodies to TGFβ1 and TGFβ2 virtually eliminates scarring in healing adult cutaneous wounds. Conversely, neutralising antibodies to TGFβ3 have no advantageous effects. Interestingly, exogenous administration of TGFβ3 also markedly inhibits scarring. Recent experiments utilizing transgenic animals add further evidence to the view that there is a profound interaction between TGFβ3 and TGFβ1 and 2. Activation of latent TGFβ requires binding at the mannose-6-phosphate receptor, transglutaminase activity and in some systems, thrombospondin or derivatives thereof. We have shown, experimentally, that antagonists of tissue type 2 transglutaminase and inhibitory thrombospondin peptides can have a minor anti scarring activity. By contrast exogenous addition of mannose-6-phosphate has a marked anti scarring activity and in addition, accelerates wound collagen deposition. Extensive experiments and development of mannose-6-phosphate as an anti-scarring agent has revealed its potential clinical utility. We hypothesize that phylogenetically wounds have been optimized for speed of healing under dirty conditions. This results in an inflammatory and growth factor overdrive. Modulation of the growth factor profile by, for example, altering the ratio of particular cytokines, eg. TGFβ3 relative to TGFβ1 and 2, can markedly improve the evolutionary side effect of scarring without adversely affecting the speed or any other parameter in the wound healing process. These observations have major therapeutic potential.

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THE ROLE OF KGF AND KGF-REGULATED GENES IN EPITHELIAL MORPHOGENESIS AND WOUND REPAIR

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Recent studies from our laboratory have provided evidence for an important role of keratinocyte growth factor (KGF) in wound repair. Thus, expression of this growth factor is highly induced during normal wound healing in mice and men (Werner et al., 1992) and wound healing abnormalities as seen in glucocorticoid-treated mice are accompanied by a defect in KGF regulation (Brauchle et al., 1995). Finally, inhibition of KGF receptor signalling in the epidermis of transgenic mice caused epidermal atrophy, dermal hyperthickening, hair follicle abnormalities and severe defects in wound reepithelialization (Werner et al., 1994). To determine potential mediators of KGF action in the skin we used the *Differential Display PCR* technology to identify KGF-regulated genes in keratinocytes. Thereby we identified two novel genes which are upregulated in these cells by KGF but not by other keratinocyte mitogens. Expression of these genes correlates with KGF expression during wound healing, suggesting that KGF might also be a regulator of these genes *in vivo*. The possible function of these genes in normal and wounded skin will be discussed. Besides these unknown genes we also identified several known growth factors which are regulated by KGF. These include vascular endothelial growth factor (Frank et al., 1995) but also members of the transforming growth factor ß superfamily such as activin. To determine a possible function of activin in the skin we analysed the expression of this factor during the wound healing process. Thereby we found a striking induction of the activin $ß_A$ chain and to a minor extent also the $ß_B$ chain within a few hours after skin injury. Wheres the $ß_A$ chain was expressed in a distinct population of cells within the granulation tissue, $ß_B$ mRNA was found exclusively in the keratinocytes of the hyperproliferative epithelium at the wound edge (Hübner et al., 1996). These results suggest multiple autocrine and paracrine activities of activin during the repair process.

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GENERATION OF STABLY TRANSDUCED HUMAN EPIDERMAL STEM CELLS.

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Systemic delivery of recombinant proteins by autologous grafts of genetically modified keratinocytes has a number of potential applications for gene therapy of inherited and acquired disorders. We have transduced human primary keratinocytes with retroviral vectors expressing a bacterial β-galactosidase (β-gal) gene or a human interleukin-6 (IL-6) cDNA under control of long terminal repeat. The highest transduction efficiency was obtained when keratinocytes were seeded for 3 days on a feeder-layer composed of a 1:2 mixture of lethally irradiated 3T3-J2 cells and producer GP+*env* Am12 cells. Efficiency of gene transfer averaged approximately 50% and 95% of clonogenic keratinocytes for β-gal and IL-6 respectively, as revealed by β-gal staining and IL-6 radioimmunoassay on isolated clonogenic cells. Exposure to G418 reduces the growth potential of transduced epidermal cells. Therefore, we avoided G418 selection in order to evaluate the life-time of transduced cells as well as the transduction of stem cells. Transgenes were stably integrated and expressed for more than 150 generations. Transduced keratinocytes secreted IL-6 at an average rate of approximately 350 ng per 10^6 cells per day during their entire life in culture. The direct demonstration of stem cell transduction was obtained by clonal analysis of IL-6-transduced keratinocytes. Clonal analysis showed that both epidermal stem cells and their transient amplifying progeny expressed the transgenes permanently. Analysis of the integrated proviral genomes showed multiple stable integrations (1 to 15) for each clonogenic cell. Cultures generated from isolated clones produced IL-6 at levels between 150 and 1000 ng per 10^6 cells per day. Secretion of IL-6 in the culture medium by cloned keratinocytes was related, although not proportional, to the number of proviral integrations. When cohesive epidermal sheets prepared from transduced cells were grafted onto athymic mice, IL-6 was detected in the circulation at levels corresponding to 6% and 95% of the values expected from IL-6 secretion *in vitro* by attached keratinocytes and detached epidermal sheets respectively. Since human keratinocytes generate cohesive sheets of autologous epithelium routinely used for the permanent coverage of large skin defects, stable transduction of epidermal stem cells creates the very attractive possibility of long-term treatment of genetic disorders, both epidermal and systemic.

Edema and cutaneous internal hemorrhage in transgenic mice overexpressing the VEGF/VPF in suprabasal epidermal cells.

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In addition to its fundamental role in tumor angiogenesis, expression of VEGF/VPF by keratinocytes has been shown to be important in several non oncogenic processes of skin such as psoriasis, wound healing and other inflammatory deseases. To further characterize the effects of VEGF/VPF in skin **in vivo** we have developed transgenic mice expressing the mouse VEGF (120) under the control of keratin K6 regulatory sequences (a 2,4 kb 5'fragment). So far, two healthy and viable founder mice have been identified. One of the founders developed scattered red spots throughout the skin. This animal was shown to be highly mosaic and transmitted poorly to its offspring. However, the transgenic progeny developed a strong phenotype characterized by swelling and erythema and die few days after birth. Histological examination of skin of transgenic animals showed increased vascularization, edema and extravased red blood cells as a consequence of augmented blood vessel permeabilization. Immunoprecipitation of ^{35}S methionine labelled proteins released to the medium of primary culture of transgenic epidermal cells demostrate a high expression of VEGF as compared to cells from control littermates. Although this lethal phenotype, as a consequence of high transgene copy number and expression, precludes extensive experimentation, we are currently generating new transgenic lines that should enable us to perform antiangiogenic drug testing and studies on the role of VEGF/VPF in skin carcinogenesis and other skin deseases.

CELL CYCLE CONTROL IN EPIDERMAL KERATINOCYTES

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The cell cycle has been shown to be controlled by at least three elements: cyclin dependent kinases (cdk's), the catalytic partners of cyclins, labile proteins that activate the cdk's, and at least two families of inhibitors of cdk kinase activity. The balance in the expression and activities of cdk's cyclins, and cdk inhibitors seems to regulate the passage of cells through the different stages of the cell cycle.

Epidermal keratinocytes are an interesting model for studying the regulation of the cell cycle *in vivo* and *in vitro*. *In vivo* proliferation is restricted in normal epidermis to the basal cell layer. As cells exit the proliferation pools and start the epidermal differentiation program, they move to the suprabasal layers and form the malpighian layer. Thus, in the epidermis the proliferative and differentiation compartments are clearly separated and can be distinguished morphologically. *In vitro*, keratinocytes can be modulated to imitate their *in vivo* behavior through the use of growth factors and the regulation of extracellular calcium.

We have studied the expression of cyclins in mouse keratinocytes after stimulation with a variety of growth factors. Cyclin D1 is induced in response to specific growth factors, such as epidermal growth factor (EGF) and keratinocyte growth factor (KGF) which do not alter the expression of other cyclins and cdk's. In collaboration with A. Glick and S. Yuspa we have also observed a marked expression of cyclin D1 upon transduction with an activated *ras* gene.

Differentiation of mouse keratinocytes was induced by switching the Ca^{2+} concentration in the culture medium from 50 μM to 1.2 mM. The high calcium concentration is known to induce morphological changes, the expression of differentiation markers and permanent cell cycle withdrawal within 24 hs of the calcium switch. In order to determine the mechanisms involved in the cell cycle withdrawal in response to calcium, we studied the expression of cell cycle proteins within 24 hs of the calcium switch. Cyclins D1 and D2 are downregulated at the steady state level, but cyclin D3 is detected well after DNA synthesis has ceased. Cyclin E is present with two isoforms that exhibit different behavior during keratinocyte differentiation. Furthermore the cdk's also show different modes of regulation. Whereas cdk2 protein levels decrease along with DNA synthesis, cdk4 and cdk6 do not undergo any appreciable changes. Expression of two cdk inhibitors was also examined. p21 was upregulated within the first 6 hours of the calcium switch but p27 was not affected.

Current studies are being pursued to investigate cell cycle regulation in keratinocytes derived from cyclin D1 null mice (obtained from Dr. R.A. Weinberg) and cyclin D1 transgenic mice (obtained in collaboration with Dr. J. L. Jorcano), and to define the role of cyclin D1 in the epidermis *in vivo*. Preliminary experiments suggest that cyclin D1 by itself can regulate proliferation in the epidermis and may be a downregulator of *ras* transformation. (Supported by grants CA 42157 and CA 57596).

RE-EXPRESSION OF LAMININ-5 BY TRANSFECTION OF THE LAMININ γ2 CHAIN cDNA CONFERS CHANGES IN CELL MORPHOLOGY, MOTILITY AND ADHESION OF JUNCTIONAL EPIDERMOLYSIS BULLOSA KERATINOCYTES.

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Herlitz junctional epidermolysis bullosa (H-JEB) is characterized by an abnormal synthesis of the laminin-5 that reduces the adherence of the diseased keratinocytes. To achieve the phenotypic reversion of H-JEB cells, we transferred the full length laminin γ2 cDNA into immortalized H-JEB keratinocytes (cell line LSV5) that carry the homozygous mutation R95X in the laminin γ2 chain. Using a polylysin-transferrin-DNA complex associated with a defective adenovirus, the transfected cells actively synthesized the recombinant γ2 chain. The polypeptide associated with the endogenous α3 and β3 chains to form mature laminin-5 that was deposited on the tissue culture substrate. Persistent expression and secretion of laminin-5 were also obtained upon infection of the H-JEB keratinocytes with a retrovirus expressing the laminin γ2 chain. Immunofluorescence analysis of stratified epithelia obtained with the transfected (LSV5R) cells demonstrated the polarized secretion of the recombinant laminin-5. Incorporation of the molecule in the basement membrane produced by LSV5R cells inoculated subcutaneously in nude mice was also observed. Re-expression of the laminin-5 enhanced the adherence of the H-JEB keratinocytes that, compared to parental LSV5 cells, presented a closer apposition of the ventral plasma membrane to the culture substrate and a flattened morphology. These observations correlated with a reorganization of focal adhesions, which in the transfectants displayed the shape and distribution observed in normal keratinocytes. Synthesis of laminin-5 also enhanced the adhesion strength of the H-JEB keratinocytes, as determined by a cell binding assay, and reduced their motility, as shown by a phagokinetic track assay. These results demonstrate that re-expression of a functional laminin-5 induces a phenotypic reversion of the diseased phenotype and open new perspectives to the study of epithelial adherence in a physiologic and pathologic context.

Growth factor alteration in transgenic animals

Chairperson: Dennis R. Roop

INVOLVEMENT OF EGFR IN POSTNATAL SKIN DEVELOPMENT AND EPIDERMAL NEOPLASIA INDUCED BY THE v-*ras*^{Ha} ONCOGENE. A.A. Dlugosz, L.A. Hansen, C. Cheng, N. Alexander, M.E. Hogan*, J.P. Sundberg*, D.W. Threadgill**, T. Magnuson**, and S.H. Yuspa. National Cancer Institute, Bethesda, MD, *The Jackson Laboratory, Bar Harbor, ME, and **Case Western Reserve University, Cleveland, OH.

The epidermal growth factor receptor (EGFR) has been implicated as a fundamental regulator of cutaneous morphogenesis and keratinocyte growth in both normal and diseased skin. Analysis of mice in which the EGFR gene has been genetically disrupted provides direct evidence that EGFR function is essential for normal hair follicle development during the perinatal period: EGFR -/- mice develop a fuzzy coat of hair with marked disorganization of follicular structures within the dermis (1-3). Since postnatal analysis of these mice is limited due to multi-organ failure and death within 2-3 weeks of birth, grafting studies were undertaken using immune-deficient nude mice as hosts. The abnormal phenotype of hair follicles seen in EGFR -/- mice was reproduced in transplanted EGFR -/- skin as well as in grafts of EGFR -/- hair follicle buds combined with either wild-type or EGFR -/- dermal cells. In contrast, normal hair follicle formation was observed when wild-type follicle buds were grafted with either wild-type or EGFR -/- dermal cells, indicating that disruption of EGFR function in the epithelial component of follicles is sufficient to produce the phenotypic changes seen in intact EGFR -/- skin. Although the initial stages of hair follicle development appeared unimpaired in EGFR -/- mice, beginning at 5 days after birth there was a progressive alteration in the morphology of hair follicles characterized by premature keratinization of both the hair shaft and inner root sheath. Consistent with these morphological observations, transglutaminase activity, keratin 6 protein, and mRNA encoding two hair-specific keratins, mHa2 and hacl-1, were detected prematurely in EGFR -/- hair bulbs. Furthermore, scanning electron microscopy revealed dramatic alterations in the structure of EGFR -/- hairs, including flattening of the hair shaft, longitudinal grooves, thinning of cuticle cells, and variation in hair shaft diameter. Although robust hair growth was observed in wild-type skin transplants from 2 to 10 weeks after grafting, hair growth in the EGFR -/- skin transplants decreased beginning at 4 weeks with complete loss of hairs in 67% of the EGFR-deficient grafts by 8 weeks post-grafting. Hair loss in EGFR -/- grafts was associated with progressive deterioration of hair follicles leading to their ultimate disappearance, the presence of a mixed inflammatory-cell infiltrate, and a residual hyperproliferative epidermis (BrdU labeling index ~3-fold higher than in wild-type grafts), corroborating results reported for transgenic mice expressing a dominant-negative EGFR targeted to the skin (4). Our findings indicate that EGFR plays a critical role in hair follicle morphogenesis, biochemical differentiation, and hair shaft formation, and is thus essential for maintaining the integrity and long-term "survival" of the follicle. In contrast, the requirement for EGFR function is less stringent, if not dispensible, for proliferation of keratinocytes in both the hair follicle and epidermis.

Data from human and animal studies suggest that activation of the EGFR signaling pathway is involved in growth regulation of a variety of epithelial tumors. The v-*ras*^{Ha} oncogene up-regulates expression of transcripts encoding four EGFR ligands in cultured murine epidermal keratinocytes, and in squamous papillomas produced by grafting these cells onto nude mice (5). The involvement of these growth factors in phenotypic responses to oncogenic Ras was tested using primary keratinocytes isclated from EGFR -/- and wild-type mice. *In vitro*, recombinant EGFR ligands (EGF, TGFα, amphiregulin, betacellulin, and HB-EGF) stimuate proliferation of wild-type but not EGFR -/- keratinocytes. Similarly, conditioned medium from v-*ras*^{Ha}-transduced keratinocytes is mitogenic for wild-type but not EGFR -/- keratinocytes. Since

the proliferative response of EGFR -/- cultures to multiple other mitogens is intact, ligands signaling through EGFR are thus likely to be the predominant keratinocyte mitogens secreted by v-*ras*^{Ha}-transformed keratinocytes. Oncogenic Ras causes characteristic alterations in the terminal differentiation program of keratinocytes. Expression of the spinous cell differentiation markers keratins 1 and 10 is blocked by v-*ras*^{Ha} in wild-type but not EGFR -/- cultures, confirming our earlier studies linking this differentiation defect in *ras*-transduced keratinocytes to EGFR activation (6). In contrast, aberrant induction of keratin 8 by v-*ras*^{Ha} is observed in cells of either genotype, indicating that this response to oncogenic Ras is not dependent on EGFR function. *In vivo*, growth of tumors produced by grafting v-*ras*^{Ha} keratinocytes onto nude mice is impaired in cells lacking functional EGFR: 26 days after grafting, the mean volume of EGFR -/- v-*ras*^{Ha} tumors was 79% lower than that of tumors produced by grafted wild-type v-*ras*^{Ha} keratinocytes. v-*ras*^{Ha} keratinocytes of both genotypes produced lesions that were grossly and histologically benign squamous papillomas, and no differences were detected in keratinocyte-specific marker expression (K1, K13, K8, loricrin, filaggrin) or incidence of apoptotic cells among tumors of different genotypes. Interestingly, in EGFR -/- v-*ras*^{Ha} tumors, BrdU-labeled nuclei were detected in several suprabasal cell layers, whereas nearly all BrdU-labeled nuclei in wild-type papillomas were restricted to the basal or first suprabasal cell layer. Our findings indicate that although Ras-induced papilloma formation is not absolutely dependent on EGFR function, inhibition of a sub-set of responses to v-*ras*^{Ha} in EGFR -/- keratinocytes suggests that certain aspects of the neoplastic phenotype are mediated by autocrine or paracrine activation of this signaling pathway.

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OVEREXPRESSION OF WILD-TYPE AND DOMINANT NEGATIVE EGFR FORMS IN THE SKIN OF TRANSGENIC MICE.

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Signaling through the EGFR is thought to be a fundamental event in the regulation of epidermal biology. Addition of EGFR ligands (EGF/TGF-α) promotes growth of cultured keratinocytes and EGF injection provokes hyperproliferation of the epidermis and delay in hair follicle development. In addition, overexpression of EGFR and its ligands has been detected in a variety of epithelial carcinomas, including squamous cell carcinomas.

To analyze *in vivo* the role of EGFR in skin, we have generated transgenic mice expressing this gene or a dominant negative (DN) mutant form under the control of the keratin K5 promotor. Animals expressing the DN receptor are small and present clear abnormalities in the structure and development of their hairs, leading to alopecia. Other epithelia, such as cornea and mammary gland, are also affected.

Histological examination demonstrates that hair follicles fail to enter into catagen stage and subsequently degenerate. These alterations lead to necrosis and disappearance of the follicles, accompanied by strong infiltration of the skin with inflammatory elements. Treatment of these animals with glucocorticoids as well as grafting their skins to immunodeficient hosts indicates that follicle degeneration is a direct effect of transgene expression that occurs in the absence of inflammation.

Biochemical experiments demonstrate that the DN receptor was effectively blocking not only the phosphorylation of EGFR but also that of HER2. In spite of these facts, s.c. injection of EGF into newborn transgenic mice produces strong epidermal hyperplasia and inhibition of follicle growth, to a degree much higher than that observed in control littermates. Biochemically, c-fos mRNA induction in the skin was also stronger and more prolonged in transgenic DN mice. Altogether, these results demonstrate that, although the DN receptor prevents EGFR phosphorylation, there is an increased effect of EGF in the skin of DN transgenic mice.

Finally, two stage skin chemical carcinogenesis experiments currently under way, indicate that these transgenic mice are rather resistant to develope tumors by this protocol.

Transgenic mice overexpressing the human wild-type (WT) receptor do not show any observable phenotype. Biochemical experiments demonstrate that the WT receptor is strongly expressed and that it is phosphorylated upon EGF s.c. injection. However, this treatment does not produce any effect on the structure or mitogenic rate (measured as BrdU incorporation) of the skin.

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TGFβ1 in epithelial growth control and carcinogenesis in the mouse

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Transforming growth factor β1 (TGFβ1), is a potent negative epithelial growth regulator and a modulator of cellular phenotype, which has been proposed to play an important role in epithelial homeostasis and carcinogenesis. TGFβ1 is induced in mouse epidermis in response to the tumour promoter, 12-0-tetradecanoyl-phorbol-13-acetate (TPA), (Akhurst et al., 1988; Krieg et al., 1991; Fowlis et al., 1992), as is its type II receptor (Cui et al., 1995), and its expression is often elevated in tumour cells *in vivo* (Derynck et al., 1987; Dickson et al., 1987; Gomella et al., 1989). However, much controversy exists as to its exact role in control of epidermal homeostasis, and its contribution, if any, to the outgrowth and phenotype of tumours *in vivo*.

As a functional approach to address the role of this growth factor in epithelial homeostasis and carcinogenesis, transgenic mice were generated which expressed TGFβ1 targeted to suprabasal keratinocytes. Either a 5 Kb bovine K10 (constitutive, two lines) or 2.2Kb K6 (inducible, 7 lines) promoter were used (Cui et al., 1995; Fowlis et al., 1996) to drive expression of latent or activated TGFβ1. The K10 transgenes were constitutively expressed. The short (2.2Kb) K6 promoter element was generally silent in untreated animals, but was induced in keratinocytes when placed in culture or, *in vivo*, in response to hyperplasia which follows topical application of the tumour promoter, 12-tetradecanoyl-phorbol-13-acetate (TPA). In one exceptional line (M5), the K6 promoter was constitutively expressed.

All the TGFβ1 transgenic lines studied showed attenuation of the basal keratinocyte proliferative response to TPA, as a consequence of inducible TGFβ1 gene expression. Surprisingly, the mice with constitutive expression of TGFβ1 showed a two to three fold increase in steady state epidermal labelling index over control mice (Cui et al., 1995; Fowlis et al., 1996). Interestingly, quiescent epidermis expressed very low levels of TβRII, which increased in response to TPA treatment, correlating with acquisition of the negative growth response to TGFβ1. Thus, the growth response of keratinocytes to TGFβ1, whether positive or negative, might be determined by expression levels of TβRII.

To address the function of TGFβ1 in skin carcinogenesis in vivo, 5 of the transgenic mice lines, (one K10 line and 4 K6 lines) were subjected to long term chemical carcinogenesis treatment with a single dose of DMBA, followed by biweekly application of TPA, for 20 weeks (Cui et al., 1996). The transgenics were more resistant to induction of benign skin tumours than controls, but the malignant conversion rate was vastly increased. There was also a higher incidence of highly malignant spindle cell carcinomas which expressed high levels of endogenous TGFβ3, suggesting that TGFβ1 elicits an epithelial-mesenchymal transition *in vivo*, and that TGFβ3 might be involved in maintenance of the spindle cell phenotype. Thus, TGFβ1 has biphasic action during multistage skin carcinogenesis, acting early as a tumour suppressor, but later enhancing the malignant phenotype. The potentially adverse effects of this growth factor should be considered when designing therapeutic interventions utilising TGFβ agonists.

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Overexpression of Bone Morphogenetic Protein-6 in the Epidermis of Transgenic Mice: Inhibition or Stimulation of Proliferation Depending on the Pattern of Transgene Expression and Formation of Psoriatic Lesions.

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Bone Morphogenetic Protein-6 (BMP-6) belongs to the family of Transforming Growth Factor-beta (TGF-beta) related growth factors. In the developing epidermis, expression of BMP-6 coincides with the onset of stratification. Expression persists perinatally but declines after day 6 pp (post partum) although it can still be detected in adult skin by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. We constitutively overexpressed BMP-6 in suprabasal layers of interfollicular epidermis in transgenic mice using a keratin 10 promoter. All mice expressing the transgene developed abnormalities in the skin indicating an active transgene derived factor. Depending on the pattern of transgene expression, the effects on proliferation and differentiation were completely opposite. Strong and uniform expression of the BMP-6 transgene resulted in severe repression of cell proliferation in embryonic and perinatal epidermis but had marginal effects on differentiation. Weaker and patchy expression of the transgene evoked strong hyperproliferation and parakeratosis in adult epidermis and severe perturbations of the usual pattern of differentiation. These perturbations included changes in the expression of keratins and integrins. Together with an inflammatory infiltrate both in the dermis and in the epidermis these aspects present all typical histological and biochemical hallmarks of a human skin disease: psoriasis.

Molecular basis of skin diseases

Chairperson: Tung-Tien Sun

EPIDERMAL FRAGILITY SYNDROMES: DISORDERS OF KERATINS AND THEIR INTERACTING PROTEINS

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Studies leading to the understanding of epidermal blistering disorders arising from mutations in keratin genes have shown us the pathological consequences of failure in structural proteins in the skin. Causative mutations in keratin genes have now been identified in a number of diverse genodermatoses. These include the different forms of epidermolysis bullosa simplex (EBS) with mutations in K5 and K14; bullous congenital ichthyosiform erythroderma (BCIE) with mutations in K1 and K10; palmo-plantar keratoderma (PPK) with mutations in K9, ichthyosis bullosa of Siemens with mutations in K2e, pachyonychia congenita types 1 and 2 (PC-1 and PC-2), focal palmo-plantar keratoderma and steatocystoma multiplex all with mutations in either K6, K16 or K17, and white sponge naevus (WSN) with mutations in K4 and K13. While most of these disorders are characterised by mutations in predictably similar cluster sites in keratin proteins, i.e. within the helix boundary peptides, the clinical phenotype of these disorders is highly divergent and focussed in each case on the cell type in skin in which the mutated keratin gene plays an important structural role; the intramolecular site of the mutation also influences the severity of the disorder (McLean and Lane, 1995). PC-2 is characterised by abnormal hair follicles and pilosebaceous cysts plus thickened nails, whilst PC-1 is characterised by absence of pilosebaceous symptoms, but presence of white plaques in the mouth (plus thickened nails). The 2 patterns reflect the different relative importance of K17 (PC-2) versus K16 (PC-1) in each of the relevant tissue types (McLean et al., 1995). The value of identifying this range of disorders lies partly in what they tell us about intermediate filament function in the cytoskeleton, including clues to filament assembly, remodelling and regulation. Heat shock model systems are now being used to disrupt mutant keratin filaments in EBS-derived cell lines (Morley et al., 1995). Perhaps most importantly, identifying the causes of these diseases allows us to characterise and classify the disorders in a more logical fashion; this is the first essential step towards evolving strategies to treat, cure and avoid these disorders in the future. We are now using knowledge gained about EBS mutation clustering in early antenatal screening for the disease.

A recognition of the molecular basis of the skin blistering diseases demonstrates a class of disorders arising from the failure of one of the many structural components in a complex tissue and subjected to physical stress. However, we have not always been able to identify keratin defect in all of the clinical samples we have attempted to analyse, and this finding is reflected in the experiences of other groups working in the field. Between some 30-60% of all samples analysed do not appear to be linked to keratin defects. The physical resilience provided by the keratin intermediate filament network is not solely dependant on the integrity of the filaments themselves, but also on the integrity of the interaction between filaments and the cell-cell or cell-substrate anchorage junctions which together with the filaments will transduce and dissipate stress across the whole tissue. It is becoming clear that as predicted a number of epidermal fragility syndromes are based not on keratin mutations but on proteins linking filaments to the plasma membrane.

We have recently studied one example of this, an unusual form of epidermolysis bullosa simplex which is linked to muscular dystrophy. By electron microscopy the skin blisters were seen to have arisen by a low-level fracture of the epidermal keratinocytes, and hemidesmosomes were abnormal and lacked the "inner plaque" structure. Immunohistochemistry demonstrated that in patients with muscular dystrophy-

epidermolysis bullosa simplex (MD-EBS), staining with antibodies to plectin and to the HD-1 antigen were consistently negative whilst all other junctional proteins we assayed appeared to be present (if in some cases attenuated). Because of the lack of plectin in MD-EBS patients, and the likelihood from our data that HD-1 and plectin may be the same or a closely-related molecule, we attempted to find a mutation in plectin by sequencing DNA from affected and normal individuals.

Plectin is a large (530 kD) widely-expressed cytoskeleton-associated protein which associates with both the intermediate filament and the actin cytoskeleton. In collaboration with Uitto and colleagues, mutations have been identified in two MD-EBS families (McLean et al., 1996; Smith et al., 1996); the affected individuals are homozygous-null for the functional protein, due to frameshift mutations leading to premature termination of the mRNA. The sequence for human plectin reveals a spectrin-like actin-binding domain in the amino-terminal part of the protein, in addition to the predicted keratin binding region in the carboxy-terminal domain of the protein (McLean et al., 1996). Thus it is possible that this large protein interacts with different cytoskeleton components, possibly even different ones in different tissues. This could explain the phenotype of 2 apparently unrelated characteristics, muscular dystrophy and EBS. In the EBS skin phenotype the lack of plectin leads to defective hemidesmosomes and failure of keratin filaments to connect to the plasma membrane, whilst in the muscle the absence of plectin leads to a deficiency in the cytoskeleton membrane attachment (presumably not hemidesmosomes since they have not been reported in muscle). Failure of the cytoskeleton membrane attachment in both cases leads to the cells reduced resistance physical stress and subsequent cell damage and structural failure within the tissue.

There will certainly be many more disorders of structural proteins uncovered in the next couple of years and one can predict, from the keratin disorders in skin, that these are likely to lead to cell fragility. This could be followed by a range of tissue-specific consequences. Structural failure of the tissue is going to be most critical where the cells are subjected to physical stress, or where the specialised nature of the cell makes it particularly dependent on cytoskeleton reinforcement. The growing awareness of molecular basis of human diseases arising from failure of structural proteins will also guide us into more productive directions for evolving new therapeutic strategies.

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THE ROLE OF EPITHELIAL SPECIFIC LAMININS IN DERMAL-EPIDERMAL ADHESION.

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The epithelial basement membrane at the dermal-epidermal junction of skin contains specialized structures termed anchoring complexes, consisting of hemidesmosomes, anchoring filaments and anchoring fibrils. Biochemical characterization of the anchoring complex has allowed the identification of several constituent molecules. The hemidesmosome is the most complex of these structures containing the transmembrane proteins integrin $\alpha6\beta4$, and collagen XVII (BPAG2), and the intracellular components BPAG1 and HD-1 (plectin). The anchoring filaments are composed of laminin 5, together with at least the exodomains of integrin $\alpha6\beta4$ and collagen XVII, and the newly identifed LAD-1/ladsin, uncin (19-DEJ-1 antigen), and a 105kDa protein recognized by patient autoimmune sera. The anchoring fibrils are composed of type VII collagen.

Mutations in the genes encoding the anchoring complex proteins found in man or induced in mice indicate a central role for laminin 5 ($\alpha3\beta3\gamma2$) (Rousselle et al, 1991) and type VII collagen in the ability of the skin epithelium to resist friction. The null phenotypes resulting from mutations in LAMA3, LAMB3, LAMC2 (encoding laminin 5 subunits) result in extreme blistering and early postnatal death. Null phenotypes resulting from mutations in COL7A1 result in extensive bllistering and dystrophic scarring. Blistering resulting from mutations in the genes encoding integrin $\alpha6$ or $\beta4$, BPAG1, BPAG2 or plectin is less severe.

Laminin 5 is unique among the laminin family in that all three subunit chains are significantly truncated within the short arm domains, and the $\alpha3$ and $\gamma2$ chains are proteolytically processed following secretion (Marinkovich et al, 1992a). We have recently identified the enzyme responsible for the processing of $\gamma2$ as BMP-1. Laminin 5 is the preferred ligand for the integrins $\alpha3\beta1$ and $\alpha6\beta4$ and can therefore efficiently mediate epithelial cell adhesion, but it cannot self-polymerize, it cannot assemble with other known laminins to form non-covalent networks, nor can it bind nidogen (Mayer et al, 1995). Therefore, it is unclear how laminin 5 functions to bind the cell surface with the basement membrane lamina densa. We have identifed covalently bonded dimers of laminin 5 with laminins 6 ($\alpha3\beta1\gamma1$) (Marinkovitch et al, 1992b) or 7 ($\alpha3\beta2\gamma1$) in tissues (Champliaud et al, 1996). Approximately 50% of the tissue laminin 5 is thus complexed. We postulate that this complex allows the required interactions with lamina densa components, primarily through the ability of the complexes to bind nidogen due to the presence of the laminin $\gamma1$ chain. We have recently observed the strong binding of monomeric laminin 5 with the NC-1 domain of type VII collagen

(Rousselle et al, submitted). We believe that this interaction provides the major connection of the dermis with the epidermis. The models we are currently testing envision a direct interaction of the epidermal cytokeratins with BPAG1 and plectin; interactions of plectin with integrin $\alpha6\beta4$, and collagen XVII with BPAG1; binding of laminin 5 by integrin $\alpha6\beta4$; and binding of laminin 5 with type VII collagen. These components are contained within the anchoring complexes. In addition, the model proposes that the laminin 5 - 6/7 complex is located in the inter-hemidesmosomal spaces, and binds the basolateral integrins $\alpha3\beta1$ and possibly $\alpha6\beta1$.

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MOLECULAR BASIS OF THE DYSTROPHIC FORMS OF EPIDERMOLYSIS BULLOSA

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Epidermolysis bullosa (EB), a group of heritable mechano-bullous disorders manifests with considerable variability in clinical severity. Also, genetic heterogeneity is evident, since both autosomal dominant and autosomal recessive forms of EB can be recognized. EB can be divided into three major categories based on the level of tissue separation, as established by diagnostic transmission electron microscopy (1). In one of these categories, the dystrophic forms of EB (DEB), the tissue separation occurs below the lamina densa at the level of anchoring fibrils, critical attachment structures extending from the lamina densa to the upper papillary dermis.

Several lines of evidence initially suggested that type VII collagen (COL7A1) was the candidate gene for mutations in DEB. First, the ultrastructural diagnostic hallmark of the dystrophic forms of EB is abnormalities in the anchoring fibrils which are morphologically abnormal, reduced in number, or completely absent by transmission electron microscopy. Secondly, these ultrastructural observations are reflected by an attenuated or absent immunofluorescence signal when anti-type VII collagen antibodies are used for staining of the skin. These morphologic observations implicated COL7A1 as a candidate gene for mutations in DEB. This suggestion was subsequently strengthened by demonstration of strong genetic linkage between the COL7A1 locus on chromosome 3p21 and both the dominantly and recessively inherited forms of DEB (2).

Recent cloning of the human type VII collagen cDNA sequences has allowed us to deduce the normal structure of type VII collagen polypeptides, the α1(VII) chain (3). Furthermore, elucidation of the intron-exon organization of the entire human type VII collagen gene (4) has provided the necessary information to undertake mutation analysis of this gene in families with DEB. In fact, specific mutations have now been disclosed in over 60 kindreds with different forms of EB (2, 5).

In the most severe, generalized Hallopeau-Siemens (HS-RDEB) type of recessive DEB, the characteristic genetic lesion is a premature termination codon mutation in both COL7A1 alleles. The major effect of a premature termination codon mutation is a dramatic reduction in mRNA abundance as a result of nonsense-mediated mRNA decay. Thus, reduced mRNA abundance and the presence of a premature termination codon for translation predict the

absence of functional type VII collagen molecules. This interpretation is consistent with the ultrastructural demonstration of complete absence of anchoring fibrils in HS-RDEB, which explains the extreme fragility of the skin in this DEB subtype.

In the less severe, mitis variant of recessive DEB, at least one and occasionally both COL7A1 alleles encode for a full-length type VII collagen polypeptide. However, these alleles contain a missense mutation or an in-frame exon skipping mutation, which can change the conformation of the protein in a manner that anchoring fibril assembly is perturbed. Thus, these attachment structures, although present, are weakened, resulting in moderately severe fragility of the skin, as observed in the mitis forms of RDEB.

In the dominantly inherited forms of EB the type of mutation detected thus far is the substitution of a glycine by another amino acid in one COL7A1 allele within the collagenous domain of the molecule characterized by the repeating Gly-X-Y sequence. Synthesis of these non-functional molecules result in a blistering phenotype through dominant negative interference. However, since type VII collagen is a homotrimer consisting of three identical α1(VII) chains, one out of eight triple helical molecules (12.5%) is entirely normal, assuming equal expression of the mutant and wild type alleles. As a result, some normal anchoring fibrils are formed, consistent with ultrastructural demonstration of thin anchoring fibrils and the relatively mild clinical phenotype in DDEB.

Collectively, the COL7A1 mutation database assembled thus far suggests that the types and combinations of specific mutations are able to predict, in general terms, the clinical severity and natural history of DDEB. Thus, the clinical severity represents a continuum in the spectrum of manifestations, and the precise nature of the genetic lesions, their positions along the type VII collagen gene, and the dynamic interplay of the two mutant alleles on the individual's genetic background will determine the precise phenotype of the patient (5, 6).

The progress made in defining the molecular basis of dystrophic forms of EB, and the genotype/phenotype correlations established from these studies, form a foundation for precise diagnosis and revised mutation-based classification, as well as for early DNA-based prenatal prediction of the genotype and genetic counseling in families at risk for recurrence. Finally, this information forms a basis for the rational design of gene therapies to counteract these devastating skin diseases in the future (7, 8).

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MOLECULAR PATHOGENESIS OF LAMELLAR ICHTHYOSIS

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TG are a superfamily of enzymes that catalyze transamidation in a wide variety of processes such as blood clotting, seminal plug formation, apoptotic coagulation, keratinization, hair formation and signal transduction. TGK mediates N^{ϵ}-(γ-glutamyl)lysine cross-linkage during formation of the cornified cell envelope (CE), a distinct and highly insoluble structure of 15 nm thickness that replaces the plasma membrane (PM) in terminally differentiating keratinocytes. This process involves the sequential cross-linking of CE precursor proteins such as involucrin, SPRRs and loricrin on the inner side of the PM. Simultaneously, the PM is replaced by Ω-hydroxyacyl-sphingosine lipids covalently bound to the outer surface of the protein CE.

We have recently shown by biochemical, enzymatic and molecular tools that keratinocyte transglutaminases (TGK) mutations cause autosomal recessive lamellar ichthyosis (LI). LI is a disfiguring congenital disorder of keratinization. Patients are born as collodion-babies or with erythroderma and develop lifelong lasting variable hyperkeratosis with large plate-like or small fine scales covering the entire body. We also found that LI is genetically heterogeneous and only in 11 out of our 27 LI families caused by defective TGK. An assay on frozen skin sections using anitbodies against dansylcadaverin to assess TGK activity in vivo combined with immunohistochemistry using B.C1 antibodies allows rapid classification. As a first step towards in vivo gene therapy, transfection of a TGK cDNA construct using the adenovirus enhanced transferrin receptor system was capable to completely repair the enzyme defect in cultured TGK-deficient keratinocytes.

The role of the integrin α6β4 in cell adhesion and the formation of hemidesmosomes.

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Hemidesmosomes are cell-matrix junctions of stratified epithelial cells, required for firm adhesion to the underlying basement membrane. A major component of hemidesmosomes is the integrin α6β4. We have generated null mutant mice for the β4 subunit by homologous recombination in embryonic stem cells. The β4 knock out mice died within 2-4 hours after birth. They showed fragility of their skin and blistering in response to minor trauma. Dermo-epidermal separations were seen over a large part of the body, with sparse and poorly developed hemidesmosomes. Histological analysis revealed many additional aberrations. In the lungs, alveoli were enlarged and often filled with exudate and the septa were thickened up to ten cell layers. The corneas were oedematous and the eyelids not closed. The tonque and the palate were fused over a considerable distance, probably as a result of wound healing and scarring.

In addition, we have identified a junctional EB patient associated with pyloric atresia (PA) in whom the integrin β4 subunit is completely absent. The α6 subunit, although reduced in amount, was normally distributed along the basement zone, as were the hemidesmososomal components BP230, BP180 and HD1. Studies with cultured PA-JEB keratinocytes showed that the α6 subunit was associated with β1 and was colocalized with vinculin in focal contacts at the end of actin stress fibers. The PA-JEB keratinocytes did not differ from normal keratinocytes in the initial adhesion to laminin-1 and laminin-5, which are both ligands for α6β1 and α6β4, but showed an increased motility on laminin-5. The results demonstrate an important role of α6β4, in both man and mouse, in the formation and maturation of hemidesmosomes and in the stable adhesion of the epidermis to the basement membrane.

Transgenic mice expressing ifn-γ in the epidermis exhibit spontaneous inflammation, perturbed epidermal homeostasis, and hypopigmentation of the hair. Joseph M. Carroll* and Fiona M. Watt- Keratinocyte Laboratory, Imperial Cancer Research Fund, London, England.

In order to more fully understand epidermal homeostasis and immunology, transgenic mice mis-expressing integrins and cytokines in suprabasal epidermis have been created. Integrins are usually only expressed in the basal layer of the epidermis, but in situations of perturbed epidermal homeostasis such as in psoriasis and during wound healing, this pattern of expression is expanded to the suprabasal layers. To determine whether or not these aberrant expression patterns are causal, mice expressing the $\alpha 2$, $\alpha 3$,$\alpha 5$,$\alpha 6$, and $\beta 1$ integrin subunits suprabasally have been made. Mice expressing solely the $\beta 1$ subunit or in combination with any α subunit exhibit epidermal hyperproliferation, hyperplasia and generalized psoriasiform phenotype. This phenotype seems to result from an initial perturbation of epidermal proliferation-differentiation. In order to better understand the spontaneous inflammation observed in these mice, transgenic mice expressing murine interferon-γ (a cytokine made by T-cells and thought to be involved in the causation of psoriasis) in suprabasal keratinocytes have been created. These mice exhibit inflammation in the skin, and basal keratinocytes in these mice are induced to express MHC-II molecules and ICAM-1. These mice exhibit varying degrees of spontaneous inflammation, flaking skin, and hypopigmentation of the skin and hair. Insights into the mechanisms of epidermal immunobiology and homeostasis will be obtained through further analysis of these mice.

Defects in lipid metabolism cause congenital ichthyosis: mutations in fatty aldehyde dehydrogenase in Sjögren-Larsson Syndrome.

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Sjögren-Larsson Syndrome (SLS) is a rare, autosomal recessive disorder in which patients have generalized, congenital ichthyosis, mental retardation and spastic di- or tetraplegia (1). Histological features of the skin include hyperkeratosis, papillomatosis, acanthosis and a thickened granular cell layer. SLS patients have a markedly reduced levels of fatty aldehyde dehydrogenase (FALDH) enzyme activity. We therefore cloned and sequenced this candidate gene, and showed that human FALDH mapped to the SLS locus on chromosome 17p11 (2,3,4). Direct sequence analysis of FALDH cDNA from SLS patients revealed a variety of mutations leading to defective FALDH enzyme. These mutations include: deletions leading to premature chain termination, a 3 bp deletion with a 21 bp insertion, a 33 bp deletion, and 4 different single amino acid substitutions due to point mutations. One of these, containing the mutation Pro315Ser, occurs at high frequency in Northern European SLS patients.

To facilitate DNA-based mutation analysis in SLS, we have characterized the genomic organization of the FALDH gene. PCR and Southern analysis of YAC clones showed that FALDH co-localizes with another aldehyde dehydrogenase gene (ALDH3). The genes were shown to be 20-70 kb apart. These enzymes differ in substrate specificity, but have extensive amino acid (61%) and coding sequence similarity. The intron/exon junctions of introns 1-8 (but not intron 9) occur at identical positions in the two genes. All FALDH introns are larger than the corresponding ALDH3 introns, resulting in an FALDH gene size of at least 28 kb. A polymorphic [CA] tandem repeat was identified in intron 8 of the FALDH gene.

Gene expression studies have been initiated to examine regulation of FALDH gene expression and gene function in epidermal differentiation. FALDH is widely expressed. Differential expression of at least three FALDH transcripts was observed by Northern blot analysis (about 2, 4 and 4.2 kb).

These transcripts appear to be due to the use of alternative polyadenylation sites. Interestingly, in epidermis, the three transcripts were equally expressed, whereas in brain the larger mRNAs were relatively more abundant than the 2 kb transcript. In addition, mRNA containing an additional coding exon derived from intron 9 sequences was detectable in all tissues examined. FALDH enzyme translated from this splice variant is predicted to have a more hydrophobic carboxyl end.

Defects in the genes for at least three lipid metabolizing enzymes, FALDH, steroid sulfatase and glucocerebrosidase, have now been associated with congenital ichthyosis. These diseases demonstrate the importance of lipid metabolism to normal desquamation and cornified layer function.

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GENERATION OF INVOLUCRINLESS MICE

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Involucrin is a keratinocyte protein, it is present in the upper layers of stratified squamous epithelia. Involucrin is a substrate of epidermal transglutaminase and along with other proteins it is thought to be a precursor of the cornified envelope. We have generated mice in which the entire coding region of the gene for involucrin was deleted. Absence of the coding region was verified by Southern blotting, absence of the mRNA and protein were verified respectively by Northern and Western blotting. After six months, the mice appear normal. Histologic sections of the skin do not reveal any abnormalities. Envelopes also appear normal under optical microscopy. We are currently investigating whether loss of involucrin is compensated by an increase in the amount of other envelope precursors.

HYPERKERATOSIS IN CYTOKERATIN KNOCKOUT MICE CAN OCCUR IN THE ABSENCE OF BASAL CELL HYPERPROLIFERATION

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Cytokeratin 10 (CK 10) knockout mice have been established using HPRT as a selectable marker to study the function of these intermediate filaments in the maintenance of epidermal integrity. These mice are used as an animal model system for the human skin disease Epidermal Hyperkeratosis (EH). As in the human disease the epidermal layer of the skin of heterozygous animals is largely thickened and shows a severe hyperkeratosis. Homozygous newborns have an extremely delicate skin upon mechanical stress and die a few hours after birth. In contrast to patients suffering from EH who show different point mutations in either CK 1 or CK 10 our mice carry a disrupted CK 10 allele. Despite this genetic difference CK 10 knockout mice represent a very good model for the human condition.

In order to find out if the defective CK 10 gene, which is expressed only in the suprabasal layers of stratified squamous epithelia, influences the proliferation of the basal layer (stem-)cells, we compared the in vivo BrdU labelling of proliferating cells in adult heterozygous mice with that of their wildtype littermates. We tested stratified epithelial tissue of different origin: tail, back, snout, paw, forestomach, oesophagus, palatinum, eyelid and ear. Interestingly only the eyelid of CK 10 heterozygotes showed a significant increase in proliferating cells. Immunofluorescence analysis revealed that in tail, snout, paw and eyelid the amount of CK 6, the expression of which is usually initiated in wound healing and during hyperproliferation, is highly elevated all over the epidermis. We suggest that the observed acanthosis as well as the hyperkeratosis of our CK 10 knockout mice are rather due to a decreased turnover of the upper cell layers than to hyperproliferation. The biochemical pathway connecting filament disruption to an altered keratinocyte cycling time is yet unknown. Currently we are constructing second step targeting vectors in order to introduce selected EH point mutations into the murine CK 10 gene.

Skin immunology

Chairperson: E. Birgitte Lane

PRIMARY CYTOKINES, EPIDERMIS, AND CUTANEOUS INFLAMMATION

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The cytokines IL-1α and β, and TNFα, were termed "primary" cytokines because their release in tissue should lead to endothelial adhesion molecule expression and cytokine production sufficient to attract and activate an inflammatory leukocytic infiltrate. IL-1 is a complex family, which comprises: 1) two agonist ligands, one of which (IL-1β) must be proteolytically processed by a specific convertase (ICE) to acquire biological activity, 2) a least two antagonist ligands, which are products of alternative splicing and bind to the IL-1R without transducing any signal, 3) a type I receptor, which induces a cascade of transcription factors including NFκB, and binds both agonist and antagonist ligands, and 4) a type II receptor, which does not transduce a signal and can be shed from the cells; this receptor binds agonist but not antagonist IL-1 ligands. Keratinocytes can produce factors from each to these four categories. To learn more about their function in vivo, we have expressed each of these under the control of the human K14 promoter. These experiments have proven that keratinocyte IL-1α can serve as a primary cytokine, and that keratinocytes can amplify inflammatory responses via autocrine activation through the IL-1R. They have further shown that the IL-1ra and the type II IL-1R, when produced by keratinocytes, inhibit local IL-1 mediated responses but not systemic responses. These data help clarify the biological role of this cytokine family in cutaneous tissues.

The Role of Neuropeptides in Mediating Inflammation in Normal and Pathologic Skin Processes

John C. Ansel, M.D.

Interactions between the nervous system and the skin are the subject of increasing interest. The cutaneous sensory nervous system not only relays sensory information from the skin to the central nervous system, but also plays an effector role in producing the skin's inflammatory response. It is now established that components of the cutaneous nervous system interact with multiple cell types in the skin to mediate actions important in skin inflammation and wound healing. There are many possible mechanisms through which the cutaneous sensory nervous system may influence skin inflammation. Sensory neurons express at least seventeen different neuropeptides. Several of these have activities or cellular localizations which suggest involvement in inflammation and tissue repair [e.g. substance P (SP), calcitonin gene-related peptide (CGRP), substance K (SK), vasoactive intestinal polypeptide (VIP)]. Substance P (SP) is the prototypic neuropeptide released from sensory C-fibers in the skin. There is much evidence that the local release of SP mediates a wide range of proliferative and inflammatory activities of the cutaneous nervous system generally referred to as neuroinflammation. The activities of SP require not only the secretion of this neuropeptide, but also the expression of the SP receptor (SPR) on local target cells and the cell surface expression of proteases that degrade neuropeptides such as neutral endopeptidase (NEP). Another important facet of nervous system-cutaneous interactions in addition to the activities of neuropeptides released by sensory fibers in the skin are the neurotrophic growth factors that mediate cutaneous reinnervation such as nerve growth factor (NGF) which is produced by the neural Schwann cells and, surprisingly, by the regenerating epidermis itself. This factor and the expression of the NGF receptor may play an integral role in nervous system-cutaneous interactions.

Substance P in the peripheral nervous system has numerous physiological actions. The peripheral actions of SP which relate to its possible involvement in cutaneous neuroinflammation fall into three main categories: 1) vasodilation and increased vascular permeability; 2) local inflammation and specific effects on the immune system; and 3) promotion of cell proliferation. In our own laboratories we have examined the actions of SP on target cells in the skin. In studies designed to examine the effect of SP on mast cell cytokine expression, we demonstrated that mast cell TNFα mRNA is selectively upregulated by SP in a dose dependent manner. SP increased secreted TNFα from both cloned CFTL 12 mast cells and freshly isolated peritoneal mast cells. We have also obtained data demonstrating that SP is capable of stimulating keratinocytes in the skin to produce specific cytokines. Addition of SP to cultured murine and normal human foreskin keratinocytes results in dose dependent increases in IL-1α, IL-1β, and IL-1ra mRNA levels that are maximal after three hours of exposure. No effects on keratinocyte TNFα or IL-8 production are seen. Interestingly, structure-function studies indicate that SP_{1-9}, but not the C-terminal fragments SP_{4-11} or SP_{7-11}, are capable of inducing keratinocyte cytokine production. More recently, we observed that CGRP can also specifically activate normal human microvascular endothelial cells to produce the neutrophil activating cytokine IL-8.

Thus, our studies and investigations by other laboratories demonstrate that there is increasing evidence that the neurologic system can modulate a wide range of inflammatory and proliferative processes. The role of the cutaneous sensory nervous system and neuropeptides such as SP in inflammation and tissue repair is complex. It is determined not only by the synthesis and secretion of neuropeptides, but also by the availability of, and interaction with, its receptor on target cells, and by the rate of neuropeptide degradation by tissue peptidases such as NEP.

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SPECIFICITY AND FUNCTION OF EPITHELIAL γδ T CELLS

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The aim of our research studies is to examine the specificity and function of the γδ T cell receptor (TCR) bearing dendritic epidermal T lymphocytes (DETC) found in murine skin. There are striking differences between these epithelial T cells and lymphoid αβ and γδ T cells. The skin γδ T cells exhibit highly restricted variable gene use, preferential pairing of TCR chains, and lack of diversity at the junctions creating a population of cells with virtually identical TCR. Generation of the DETC appears to be restricted to a discrete stage in development. These characteristics suggest that the DETC may have a specialized function in the skin and may play a role in immune system development and regulation. We have postulated that the role of γδ T cells with invariant TCR may be to recognize damage-induced self antigens on neighboring cells expressed after a variety of insults.

Analysis of epidermal tissue sections revealed that the γδ DETC reside in intimate contact with keratinoctyes, suggesting the possibility of immunological interactions between the two cell types. We have demonstrated that DETC recognize and respond to stressed keratinocytes in vitro and that this recognition is mediated by the T cell receptor. A possible consequence of the recogition of damaged keratinocytes by the DETC is production of cytokines which may help in the recovery process. We have recently described the inducible expression of keratinocyte growth factor (KGF) by activated DETC. KGF is a recently described member of the fibroblast growth factor family with specific mitogenic activity for keratinocytes. Strikingly, γδ T cells located in lymphoid organs do not express KGF, while γδ T cells found in the intestinal epithelium also produce KGF in an inducible manner. No lymphoid or epithelial αβ T cells appear capable of producing KGF. This raises the possibility that a function of the epithelial γδ T cells is to recognize and respond to damaged neighboring cells by secretion of a tissue specific factor which allows for timely and localized proliferation.

Mechanisms must exist to coordinate and modulate the activities of the αβ and γδ T cell subsets. We have recently demonstrated that the DETC can signal migration of non-resident cell types into the epidermis by the secretion of a distinctive panel of chemokines. We have determined that expression of MIP-1α, MIP-1β, RANTES, and lymphotactin was inducible in DETC while MCP-1 production could not be detected. Strikingly, lymphotactin was the most abundant chemokine produced by activated DETC. This currently unique member of the C chemokine family is the only lymphocyte selective chemoattractant described so far. Activated intestinal epithelial T cells expressing γδ TCR also inducibly expressed lymphotactin while spleen γδ T cells did not. T cell migration induced by culture supernatant from stimulated DETC was strongly reduced in the presence of neutralizing anti-lymphotactin antibodies. Collectively, these results indicate that epithelial γδ T cells play a multifaceted role in the maintenance of epithelia homeostasis in health and disease.

The epithelial tissues are the major protective barrier for mammals and are constantly exposed to a variety of deleterious agents. The epithelial γδ T cells could potentially provide a mechanism of broad defense during development before the exquisitely specific αβ T lymphocytes arise. In the adult, surveillance for stress-induced self antigens on neighboring cells and localized production of epithelial specific growth factors and chemokines may be a specialized function of epithelial γδ T cells, allowing them to provide a first line of defense against damage or malignancy.

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POSTERS

MOLECULAR BIOLOGY OF SKIN AND SKIN DISEASES

Mechanisms of Human Keratinocyte Differentiation Rhoda M. Alani, Karl Münger; Harvard Medical School, Boston, MA.

Stratified epithelia undergo a constant process of cellular differentiation. In these tissues, basal cells comprise the active proliferative layer of the stratified epithelium with cells progressing to a point of terminal differentiation as they migrate through the stratified layer of cells. The processes of cellular proliferation and terminal differentiation are tightly coupled. While many molecular markers of terminal differentiation have been well characterized, relatively little is known about the molecular controls that link the processes of cellular differentiation with the cell cycle.

Antimitotic signals that are necessary for the maintenance of balanced cell growth may be provided by modulating G1-phase cyclin-dependent kinases. Several proteins have been described that function to stoichiometrically inhibit these cyclin/cdk complexes. Among these inhibitory proteins is the cyclin/cdk associated protein, p21. p21 has been shown to be induced by the tumor-suppressor gene, p53, during DNA damage. Additionally, p21 has been implicated in the regulation of G1 arrest during cellular differentiation.

We have developed a simple reproducible system to study human keratinocyte differentiation. Neonatal foreskins are harvested for keratinocyte cultures and induced to differentiate in a calcium-enriched medium. Preliminary experiments have allowed us to characterize this system with regard to alterations of cell cycle controls during differentiation. We have been able to determine that, like in other mammalian differentiation systems, human keratinocyte differentiation is associated with the rapid induction of the cell cycle inhibitory protein, p21. This induction of p21 expression occurs in a p53-independent fashion, and cyclin A, cyclin E, and p21-associated kinase activity is inhibited during this process. We have also analyzed additional cell cycle regulatory protein expression and function during human keratinocyte differentiation including the G1 cyclins, cdks, and cyclin/cdk inhibitors.

Since our initial experiments identified p21 as a potential molecular link between cellular differentiation and cell cycle control, we have evaluated the transcriptional control of p21 during keratinocyte differentiation. We used deletion mutants of the p21 promoter fused to a reporter construct to map transactivation activity during keratinocyte differentiation. Through these experiments we have been able to localize a region of the p21 promoter that is important for the induction of p21 expression during human keratinocyte differentiation. Additionally, we have been able to identify an oligonucleotide sequence contained within this region that is able to bind to nuclear proteins that are induced during human keratinocyte differentiation. We are currently in the process of a performing a series of experiments to isolate and identify this protein.

CONTROL OF MELANOGENESIS BY PARACRINE FACTORS IN BIG MOUSE MELANOMA CELLS.

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Current evidence suggest that melanogenesis is controlled "in vivo" by epidermal paracrine modulators. We have analyzed the effects of TGF-β1, a cytokine produced by keratinocytes, on basal melanogenic activities in B16F10 cells. This cytokine elicited a concentration-dependent decrease in basal tyrosine hydroxylase and dopa oxidase activities of B16 cells to less than 30% of the control values at saturating doses measured at 48 h after treatment. In contrast, no effect could be detected for dopachrome tautomerase activity in the same conditions. The effect was mediated by high affinity receptors, with a 50% inhibition at about 10^{-11} M. The inhibition affected both the dopa oxidase activity associated to TRP-1 and tyrosinase at a similar extent, since the activity of both isoenzymes were decreased under SDS-PAGE separation. The effect of TGF-β1 was rapid, noticeable 1h after the addition of TGF-β1, and the inhibition reached maximal values after 6 h of treatment. The decrease in the enzymatic activity was concomitant to a parallel decrease in the cell contents of the tyrosinase and TRP-1 proteins as shown by western blot and detection with the specific antibodies αPEP-7 and αPEP-1 respectively. Using northern blot analysis, we could only observe after 48 h of treatment a small decrease in the expression of tyrosinase gene while the expression of TRP-1 and TRP-2 were not affected. TGF-β1 lowed the levels of tyrosinase and TRP-1 proteins by increasing their rate of degradation as shown by the decreases in the half-life for tyrosine hydroxylase and dopa oxidase activities detected after the treatment with the cytokine (from 3.2 h for the control cells to values near 1.6 h for TGF-treated cells). We suggest the unstabilization of TRP-1 and tyrosinase enzymes is the main mechanism responsable for the inhibitory effect of TGF-β1 on the melanogenic activities of B16F10 cells.

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Differentiation of β_1 integrin-deficient embryonal stem cells into keratinocytes is prevented in vitro but not in vivo

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β_1 integrins are known to regulate terminal differentiation and morphogenesis in the adult epidermis. We have investigated their role in the embryonic development of keratinocytes by comparing the differentiation of wild-type and β_1-null mouse embryonal stem (ES) cells. By 12-15 days in culture, differentiation of embryonic and simple epithelial cells occurred in both ES cell populations, as detected by expression of keratins 8, 18 and 19. From 21 days, expression of keratins 1, 10 and 14 and of the cornified envelope precursor involucrin indicated that the wild-type cells had differentiated into keratinocytes. In contrast, keratinocyte markers were not expressed in β_1-null cultures. The β_1-null cells failed to express the α_2, α_3 and α_6 integrin subunits on the cell surface, consistent with the association of these α subunits with β_1. β_4 expression was also reduced in the β_1-null cultures, possibly reflecting the fact that $\alpha_6\beta_4$ is highly expressed on keratinocytes. Although β_1-null ES cells failed to undergo differentiation into keratinocytes in vitro, they did form keratinocyte cysts expressing keratins 1, 10 and 14 and involucrin when allowed to form teratomas by subcutaneous injection in mice. Since the expression of extracellular matrix proteins is severly impaired in the β_1-null ES cells in vitro but not in the teratomas, we speculate that the β_1-deficient cells are able to form a basement membrane in vivo, but not in vitro, and that in its presence the $\alpha_6\beta_4$ integrin might be able to fulfil the role of β_1 integrins in regulating terminal differentiation.

IL-1 INDUCES TRANSCRIPTION OF KERATIN K6 IN HUMAN EPIDERMAL KERATINOCYTES

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Keratinocytes respond to injury by releasing IL-1, which serves as a signal to surrounding cells. Among the autocrine consequences of IL-1 release is production of additional cytokines and their receptors, as well as an alteration in the keratinocyte cytoskeleton caused by the induction of keratin K6 and K16 expression, as described here. Using DNA-mediated cell transfection, we have analyzed the molecular mechanism regulating keratin K6 expression by IL-1. The induction of K6 transcription is dose and time dependent and inhibitable by IL-1 neutralizing antibodies. Other keratin gene promoters tested are not induced by IL-1. Only confluent keratinocytes respond to IL-1. The addition of EGF, simultaneously with IL-1 synergistically augments the effects of IL-1 in HeLa cells. Using a series of deletions and mutations, we have identified the DNA element required for the induction by IL-1 and found that it contains four C/EBP-like binding sites. The two AP1 sites and the NFκB site upstream from the element are dispensable for IL-1 signaling, although they may convey the synergistic effect of EGF. From these data we conclude that IL-1 initiates keratinocyte activation not only by triggering inflammatory signaling events, but also by inducing directly the synthesis of keratin K6, a cytoskeletal marker of activated keratinocytes. IL-1 accomplishes this by activating transcription factors that bind a complex of four C/EBP-like sites and are capable of synergistic interaction with additional signaling mechanisms.

INTEGRIN NORMALIZATION PARALLELS BASEMENT MEMBRANE FORMATION AND EPIDERMAL HOMEOSTASIS IN NUDE MOUSE GRAFTS OF HUMAN KERATINOCYTES

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The changes of integrin patterns and formation of basement membrane in correlation to epidermal structure were investigated in transplants of human keratinocytes onto nude mice and the relevance as tissue regeneration model was evaluated. Analysis of epidermal markers and proliferation by immunofluorescence revealed a marked tissue activation around the first four days, declining rapidly until day seven, and nearly complete epidermal normalization after two weeks. The first stage was characterized (1) by depolarized distribution of the integrin chains α3, α6, αv and ß1, while ß4 showed a nearly exclusive and α2 (although very diffuse) a preferential basal location, (2) a high rate of keratinocyte proliferation (BrdU-labelling) also in suprabasal position and (3) only marginal deposition of most basement membrane (BM) components except laminin-5 and nidogen being strongly expressed already at day one and four, respectively. Tissue consolidation between day seven and eleven was signified by distinct staining for basal type keratins (K5 and 14) and pericellular arrays of integrin chains α3 and ß1, both largely restricted to the basal layer, (2) a dramatic decrease of proliferating cells seen exclusively in basal position, (3) a marked linear distribution of BM-components such as laminin-1 and type IV collagen and (4) colocalization of integrin α6ß4 and bullous pemphigoid antigen. Simultaneously, hemidesmosomes and longer stretches of lamina densa developed according to electron microscopy. At two weeks the basement membrane was continuous. This coincided with the regular distribution of the integrins α2ß1, α3ß1 and α6ß4 as well as early (suprabasal keratins, K1 and K10) and late differentiation markers (involucrin, filaggrin, loricrin), while proliferation continued at a nearly constant level. However, decoration of the BM-zone by type VII collagen was weak but increased towards three weeks together with the ultrastructural appearance of anchoring fibrils below the lamina densa. These criteria imply that finally epidermal homeostasis was accomplished in this transplantation system.

TGF-β_1 as a modulator of epithelial differentiation in transformed mouse epidermal keratinocytes

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TGF-β_1 induced an epithelial-mesenchymal transdifferentiation, and a poorly differentiated tumor phenotype, in transformed mouse epidermal keratinocytes of the squamous carcinoma cell line PDV (Caulín C et al., Cell Growth & Differ., 6: 1027-1035, 1995). PDV cells escaped to the strong inhibition of growth exerted by this growth factor in normal epithelial cells. On the contrary, nontumorigenic immortalized keratinocytes (MCA3D) or keratinocytes derived from benign papillomas (PB), when treated with TGF-β_1, ceased to proliferate, senesced and died. MCA3D immortalized keratinocytes trasnsfected with a Ha-ras oncogene (AT3) also responded to TGF-β_1 by a conversion to a fibroblast-like phenotype. AT3 cells were tumorigenic and induced squamous cell carcinomas upon transplantation in nude mice. However, MCA3D cells transfected with a normal Ha-ras gene behaved as the parental cell line.

TGF-β_1-treated carcinoma cells that co-expressed vimentin and keratins and had reduced E-cadherin expression, displayed a mixed phenotype, intermediate between epithelial and mesenchymal, and showed increased invasive and metastatic characteristics. On the other hand, transformed cells susceptible to phenotypic modulation by TGF-β_1 responded to the growth factor by increasing the secretion and activity of urokinase (u-PA), a proteinase involved in invasiveness and cell migration, whereas normal growth-inhibited cells did not respond.

These results suggest that: 1) transformed epidermal cells acquire a more plastic phenotype susceptible to modulation by TGF-β_1; 2) this growth factor can act as a stimulator of malignant progression in epidermal carcinogenesis.

c-Myc REGULATES DIFFERENTIATION OF HUMAN KERATINOCYTES

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Resembling the physiology of the epidermis, human keratinocytes undergo terminal differentiation when placed in suspension (Adams and Watt, 1990). After 24 hours most cells express involucrin, a marker of terminal differentiation. After 5 hours c-Myc mRNA is down-regulated, whereas Mad mRNA is up-regulated (Gandarillas and Watt, 1995).

In order to explore the role c-Myc plays in keratinocyte differentiation, we have infected human primary keratinocytes with retrovirus bearing c-mycER^{TM} chimaeric protein. ER^{TM} is a mutant oestrogen receptor that responds to the synthetic steroid 4-hydroxytamoxifen (TMF) but not to the natural hormone and allows us to regulate myc activity (Littlewood et al, 1995). TMF-activation of c-mycER^{TM} in the infected cells caused an imbalance in the ratio growth/differentiation that was not observed in the absence of TMF. Furthermore, the effect was reverse when cells were infected with a chimaeric ER^{TM} construct bearing the non-functional c-myc deletion mutant D106-143, thus indicating that integrity of the protein was required. The results show that c-myc plays an important role in keratinocyte growth and differentiation..

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Expression and regulation of activin *in vitro* and during wound repair of the skin

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Recently we analysed the expression pattern of activin and its binding proteins in normal and wounded skin. We demonstrated a large induction of activin mRNA expression one day after skin injury. In contrast, inhibin mRNA was hardly detectable. 13 days after wounding expression of activin β_A mRNA had returned to the basal level, whereas expression of activin β_B mRNA was still high. All known types of activin binding proteins were expressed in normal and wounded skin. However, no significant induction of these genes was seen during the repair process. *In situ* hybridization studies revealed expression of activin β_A in the granulation tissue below the wound and activin β_B in the hyperproliferative epithelium at the wound edge and in the migrating epithelial tongue. To identify possible mediators of the activin induction during skin repair, we have now analysed the regulation of activin expression in cultured keratinocytes and fibroblasts. Here we show that activin mRNA is low in quiescent keratinocytes and fibroblasts but is strongly induced upon serum stimulation and by isolated serum growth factors like transforming growth factor β, platelet derived growth factor, and epidermal growth factor. Furthermore, we also found a strong activin induction *in vitro* by cytokines such as interleukin (Il) 1β and tumor necrosis factror α (TNF-α). Little is known about the expression pattern and regulation of these pro-inflammatory cytokines in the wound tissue. To determine if these cytokines could be activin inducers *in vivo,* we investigated their temporal and spatial expression pattern during skin repair. We found a strikingly increased expression of Il-1α, Il-1β, and TNF-α within a few hours after skin injury. Expression started to decline 5 days after wounding. *In situ* hybridization studies revealed macrophages and polymorphonuclear leukocytes as the most important sources of pro-inflammatory cytokines in skin wounds. In conclusion, growth factors derived from the serum upon hemorrhage are likely to initiate the large induction of activin expression after skin injury, whereas the pro-inflammatory cytokines can be responsible for the prolonged expression at later stages of the repair process.

HIGHLY PERSISTENT LABEL-RETAINING CELLS IN THE HAIR FOLLICLES OF MICE AND THEIR FATE FOLLOWING INDUCTION OF ANAGEN.

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We have identified in the hair follicles of mice, a highly persistent subpopulation of keratinocytes that remains for at least 14 months following labelling. To identify these cells, BDF_1 female mice were injected subcutaneously with 10 μCi of [^{3}H]thymidine (specific activity 25 Ci/mmol) in 50 μl twice daily for 3 days beginning the third day after birth. The mice were weaned at 3 weeks and were housed under standard conditions for 14 months after which they were anesthetized and were plucked by enmeshing the hairs with plastic dressing and then removing the dressing. Light microscopy confirmed that essentially all of the follicles were in telogen at the time of plucking. Every 6 hours for 48 hours, groups of 3 to 4 mice were euthanized, pieces of the dorsum were fixed in formalin for paraffin sections with the hair follicles oriented longitudinally, and light microscopic autoradiography was performed. In unplucked mice no labelled cells were found in the interfollicular epidermis and labelled cells in the hair germ, the sebaceous gland and duct, and the hair canal were very rare. The index of labelled cells was 2 percent in the follicle of the secondary follicle and was 5 percent in the primary follicle. The labelled cells were located at Spain abstract the confluence of the primary and secondary follicles and at the outer aspect of the primary follicle 4 to 5 cells from the base. Serial sections confirmed that the follicle of the primary follicle was continuous with the "bulge region", the site of attachment of the arrector pilorum muscle. Plucking stimulated growth of the hair follicles such that by 18 hours, the follicles had entered anagen 1 and by 36 hours had entered anagen 2. At no time after plucking were labelled cells found in the germ; they remained in the mid-follicular region. These observations support the hypothesis that the highly persistent labelled cells are stem cells. These observations also suggest that the follicle germ enters anagen autonomously without migratory influx of stem cells.
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Functional role for the Retinoblastoma family of proteins in epidermal differentiation

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The involvement of the pRb related proteins in the physiology of human epidermis has been studied. Transfection experiments in a human keratinocyte cell line indicate that the overexpression of certain combinations of these proteins can specifically promote different stages of the epidermal differentiation process. In particular pRb and p107 co-operate to drive the early stages of the process (keratin K10 expression) while p130 appears to be involved along with pRb and p107 in later stages (involucrin expression). However, among these proteins only p107 seemed to promote the cell cycle arrest of these cells. The involvement of these protein in this process is also reinforced by their specific localisation in human skin in situ analysed by immunofluorescence and Western Blotting of human keratinocytes during in vitro differentiation experiments.

EXPERIMENTAL MODEL OF LOCAL ADMINISTRATION OF GROWTH FACTORS IN REEPITELIZATION

(Authors: Esther Pascual, Sonia Sanz, Xavier Santos, Gonzalo Bilbao). Hospital del Aire - Madrid

The most recent studies relate the cicatrization etiopathogeny with many growth factors as TGF-b, PDGF, IGF-1 and NGF. They consider that EGF, KGF, b-FGF y TGF-α are specially implicated in reepitelization.

For this reason we suppose that the administration of these growth factors in equal or superior concentrations to physiological ones, in a continuous topic way over a partial-thickness skin wound, could help qualitatively and quantitatively the reepitelization.

To confirm this hypothesis we have developed an experimental model that allows the administration of the growth factors, maintaining continuously the dose in the factor maximun efficacy average.

In this model we use Wistar rats (200-250 g males) that are anaesthetised via intraperitoneal for the creation of a partial-thickness skin wound of 2x2 cm^2, on the interscapular area, with a handy dermatomo. Then we place a hydrocolloid aposite (Geliperm) saturated with a phosphate buffered solution of the growth factor (EGF 10 μg/ml; KGF 3.3 ng/ml; b-FGF 1 μg/ml). A plastic film is sprayed over the hydrocolloid and this is covered with a tied dressing.

Finally, we use a morphometer to valorate reepitelization. With this we can quantifie:
- the thickness of the whole epithelium (from basement membrane to corneum stratum),
- the inflamatory infiltrated/area.

We have previously done a chronological study of reepitelization with 27 rats (nine groups of three rats each one, taking samples from the first to the ninth day), and, after verifying that 90% of rats had reepitelizated the sixth day, we have established four groups of study:
- control (20 rats)
- EGF (20 rats)
- KGF (20 rats)
- b-FGF (20 rats)

We take samples at 3rd and 5th day (10/10). We follow the same process for each factor.

The preliminary results with EGF are presented.

CHROMOSOME 9p DELETIONS AND p16 MUTATIONS IN MALIGNANT MELANOMA

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Malignant melanoma (MM) is a fatal form of skin cancer whose incidence is rising steadily at a rate of 5% per year. Around 10% of MM cases are familial, often in association with dysplastic or clinically atypical nevi. An increased incidence of other kind of cancer such as pancreas adenocarcinoma has been described in these patients.

Recently, the *p16* gene (also called *CDKN2, CDK4I* or *MTS1* gene) has been cloned from chromosome 9p21, which is an inhibitor of cyclin-dependent kinase 4. *p16* has been found to be rearranged, deleted or mutated in the majority of tumour cell lines indicating its role as a tumour suppressor gene. The melanoma predisposition gene (*MLM*) has been mapped to 9p21 through linkage and LOH analysis. Different studies have been done to prove whether they are the same gene providing conflicting evidence: while Hussusian et al. have described potentially inactivating mutations in nearly 75% of families, Kamb et al have only found *p16* mutations in less than 20% of families.

The aim of the present work was to elucidate the role of *p16* in sporadic and familial MM. We have studied 12 microsatellite markers in 9p in 54 sporadic cases to determine and delimitate the presence of 9p deletions. Also we have studied through SSCPs analysis and sequencing of abnormal *p16* bands 49 sporadic MM tumours, 7MM families and 9 patients with melanoma and another associated malignancy (5 patients with more than one primary MM, 1 patient with pancreatic adenocarcinoma, 1 patient with mesothelioma and 1 patient with bladder carcinoma).

Nearly 50% of the tumours analysed showed LOH at 9p. Several clinicopathological types of MM had deletions of 9p including two of the three in situ tumours studied, suggesting that a tumor suppressor gene at 9p21 is involved in the early stages of the disease. Microsatellite deletions in some tumors do not involve the *p16* or *p15* (a *p16* homologous gene that maps a few kilobases from *p16*) genes and place the *MLM* locus closer to the centromere than these genes.

We have detected a missense mutation (Asp 145Asn) in 3 metastases from a patient who has a normal *p16*. One of these metastases also presents LOH for this region.

Only 1 MM familiy presented a mutated *p16*. Several members have a frameshift mutation due to a one base pare deletion at nt 351.

In more than 4% of the samples studied we have found a previously described polymorphism (Val140Thr).

Our results suggest that even though *p16* is *MLM* gene in some predisposed cases, the rare second hit mutations in sporadic tumours and low proportion of *p16* mutations in predisposed individuals implies that another tumour suppresor gene(s) exists in 9p21 involved in the early stages of melanoma development.

IN VITRO ANALYSIS OF KERATINOCYTES FROM TRANSGENIC MICE EXPRESSING INTEGRINS IN THE SUPRABASAL EPIDERMAL LAYERS

M. Rosario Romero, Joseph M. Carroll and Fiona M. Watt

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Integrin expression is normally confined to the basal epidermal layer. However, when epidermal homeostasis is perturbed, for example during wound healing, in psoriasis and in some squamous cell carcinomas, the receptors are also expressed by suprabasal, differentiating cells. The human involucrin promoter has been used to express the human integrin subunits α_5, α_2 and β_1 in the suprabasal epidermal layers of transgenic mice. Mice expressing α_5 or β_1 alone, $\alpha_2\beta_1$ or $\alpha_5\beta_1$ exhibit epidermal hyperproliferation, altered keratinocyte differentiation and skin inflammation, all of which are features of psoriasis. In order to understand the mechanism by which suprabasal integrin expression affects these processes, we have been studying the properties of keratinocytes cultured from control and transgenic mouse epidermis. The functional status of the transgenic integrins has been investigated in assays of cell-cell and cell-extracellular matrix adhesion. The capacity of cells from the transgenic mice to proliferate and undergo terminal differentiation has also been examined. These studies allow us to assess the extent to which the abnormalities of the transgenic epidermis are inherent to the keratinocytes or dependent on interaction between the epidermis and cells of the immune system.

EPIDERMAL KERATINOCYTES POSSESS VERY HIGH CAPACITY TO TAKE UP AND METABOLIZE EXOGENOUS ALL-*TRANS*-RETINOIC ACID. R. Keith Randolph and Marcia Simon, Living Skin Bank, University Hospital, Department of Oral Biology and Pathology, and Department of Dermatology, SUNY, Stony Brook, Stony Brook, NY.

The uptake and metabolism of exogenous all-*trans*-retinoic acid (RA) by cultured human epidermal keratinocytes was examined. Exogenous RA presented to keratinocytes in physiological form bound to albumin was avidly taken up. Intracellular concentrations of RA were 20 to 50-fold higher than the RA concentration of medium and were linear over a medium RA concentration range extending from 1 nM to 1 μM. The rate of RA uptake and metabolism was very rapid. Intracellular RA concentration peaked by 1-2 h and decreased steadily thereafter with a half-life of 6 h. The kinetics were the result of metabolism rather than exchange or excretion of RA back into the medium. 80% of intracellular RA was metabolized to unidentified compounds more polar than 4-hydroxy-, 4-oxo-RA, or glucuronides. 80% of these unidentified polar metabolites were recovered in the medium; the balance was retained by cells. The production and clearance of the very polar retinoid metabolites was inhibited 50% by 10 μM ketoconazole.

10-20% of intracellular RA was converted to 3,4-ddRA. The conversion of RA to 3,4-ddRA was proportional to medium and cellular RA concentration and was not affected by ketoconazole.

The results suggest that uptake and metabolism of RA by "basal" keratinocytes has potential to shield differentiating suprabasal keratinocytes from RA in plasma.

List of Invited Speakers

Workshop on

MOLECULAR BIOLOGY OF SKIN AND SKIN DISEASES

List of Invited Speakers

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MOLECULAR BIOLOGY OF SKIN AND SKIN DISEASES

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