Instituto Juan March
de Estudios e Investigaciones

28 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Human and Experimental Skin Carcinogenesis

Organized by

A. J. P. Klein-Szanto and M. Quintanilla

A. Balmain
F. F. Becker
J. L. Bos
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D. E. Brash
A. Cano
C. J. Conti
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The lectures summarized in this publication were presented by their authors at a workshop held on the 9th through the 11th of May, 1994, at the Instituto Juan March.

Depósito legal: M. 20.320/1994
I. S. B. N.: 84-7919-528-2
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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HUMAN AND EXPERIMENTAL SKIN CARCINOGENESIS

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INTRODUCTION

M. Quintanilla

Because of exposure to UV and other carcinogens the skin is one of the most frequent sites of tumors in humans. Thus, the frequency of non-melanoma skin cancer has incresed significantly during the last decade in many parts of the world.

Advances in oncogene, tumor suppressor gene and growth factor research have had an enormous impact on our knowledge of tumor pathogenesis. In skin cancer, the study of animal model systems such as the classical model of chemical mouse skin carcinogenesis has provided a more precise identification of genetic and epigenetic changes characteristics of the different stages of tumorigenesis: initiation, promotion and malignant progression. In the mouse model, as occurs in human cancer in general, genes such as H-ras and p53 have been found to be critical targets for cancer development. However, differences exist when the mouse and the human skin systems are compared. Thus, for example, in mouse skin carcinogenesis, the inactivation of the p53 gene has been associated with progression from papillomas to carcinomas whereas p53 mutations appear to occur early during tumor development in human UV-induced skin cancer.

Recent progress in understanding the role of critical genes in carcinogenesis has come from the generation of transgenic and knockout mice. Targeted expression of normal and mutant genes to the epidermis of transgenic mice have been carried out by using different keratin promoters. This strategy has produced animal models that mimic discret stages of skin carcinogenesis.

Another crucial point that underlines all cancer pathogenesis is the elucidation of the biochemical pathways altered by genetic events, the unregulated signals that lead to selective clonal expansion and resistance to terminal differentiation of damaged cells. At this respect, the knowledge of the extracellular factors and intracellular mechanisms involved in the regulation of keratinocyte proliferation and differentiation are important not only to understand the biology of the epidermis but also for carcinogenesis. In addition, the study of the molecular basis of the phenotypic alterations associated with malignant conversion that allow transformed cells to acquire invasive and metastatic properties is a fundamental clue for our understanding of cancer and for clinicians interested in developing therapeutical approaches.

This workshop has brought together the international experts in the field of skin carcinogenesis in order to discuss the major advances on the cellular and molecular biology of this type of neoplasia. The organizers are very thankful to all the participants whose scientific contributions and discussions have made the success of this meeting possible.

I. Introduction to Carcinogenesis and Molecular Carcinogenesis of Skin Neoplasia

Frederick F. Becker, M.D.

"Recent Advances and Unanswered Questions in Carcinogenesis"

Although the field of carcinogenesis includes the study of biological and physical agents, it is has utilized the series of events following exposure to chemical carcinogens and co-carcinogenic agents as its dominant paradigm. This approach has had both salutary and potentially detrimental side effects. The former has allowed us to recognize the sequential nature of the process by which a normal cell evolves through a number of stages, eventually developing the phenotype of a cancer cell. It has also allowed us to identify the fact that multiple agents can be utilized jointly or sequentially in this process, many of which are chemically and functionally independent, yet force the sequence onward through these phased changes. When one compares different organs and tissues during carcinogenesis the commonality of these alterations has become increasingly evident with the recognition that many molecular, and cellular as well as architectural events are fundamentally similar. For example, that a stage of reversible alteration may lead to a stage of irreversible alteration (autonomy), and that this latter stage is the true predecessor of progression to malignancy has been recognized in almost every organ examined and regardless of the carcinogenic challenge.

Among the potential factors that may contribute to these sequential events, the oncogenes/suppressor genes and endogenous events such as auto-oxidation, have dominated recent literature and have, in some sense, occluded our critical thinking as to their proportionate roles in the sequence; and perhaps the possibility that other forms of intervention, stimuli and inhibitors may be significant. Thus, while in some systems evidence is mounting that activated oncogenes, are expressed in such tissues and may play an obligate role, we frequently ignore the large percentage of tumors, even within a specific organ system, that do not demonstrate these activated oncogenes, usually explaining the negative finding as resultant of failed technology. Broad speculative theories based on the presence of activated oncogenes such as a putative growth advantage in those cells expressing these agents, are not always supported by a critical analysis of the literature but the latter is not always noted.

Other contributing factors such as the influence of chronic infections are known to participate in some systems but their role in the broad based area of carcinogenesis has not been generally accepted. However, with increasing evidence of the potential role of helicobacter in human and animal tumors, it may be necessary for us to reevaluate prior results. Other areas of discussion will focus upon the gap in our information concerning crucial, endogenous processes such as those leading to promotion and progression.

It will be the goal of my presentation to raise questions and challenge assumptions that may have been too readily accepted into the paradigm for carcinogenesis.

Skin carcinogenesis: Characteristics, mechanisms, and prevention.

Thomas J. Slaga, Ph.D., Science Park - Research Division, University of Texas M.D. Anderson Cancer Center, P.O. Box 389, Smithville, TX 78957

The events leading to skin cancer development in mice can be operationally divided into at least three major stages: initiation, promotion, and progression. The first stage is thought to involve an irreversible alteration in some aspects of growth control and/or differentiation, possibly by activating the Ha-*ras* oncogene in epidermal stem cells. Many different chemical carcinogens as well as ultraviolet light when given at a subcarcinogenic dose(s) can effectively initiate the development of skin tumors. Most of the chemical carcinogens that are used as initiators must first be metabolized by the mixed-function oxidase system to electrophilic intermediate(s) before they are active. A number of agents based on their mechanism of action have been found to inhibit skin tumor initiation. In general, agents that inhibit the metabolic activation of carcinogens, increase the detoxification of carcinogens, scavenge the electrophilic intermediates and free radicals from carcinogens, and inhibit cell division, have been found to counteract skin tumor initiation. In an attempt to find highly specific and effective inhibitors of the initiation stage of carcinogenesis, we decided to investigate mechanism-based irreversible inactivators (suicide inhibitors, SI) of carcinogen metabolism. We first prepared and tested a series of SI compounds as suicide substrate inhibitors of the P450 isozymes responsible for activating polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (B[a]P) and 7,12-dimethylbenz(a)anthracene (DMBA). Based upon analysis of the inhibition of B(a)P metabolism in liver microsomes, we further tested 1-ethynylpyrene (EP) and 1-vinylpyrene (VP) as inhibitors of B(a)P and DMBA covalent binding to epidermal DNA and tumor initiating activity in mouse skin. EP in a dose-dependent manner was found to be a very effective inhibitor of both DMBA and B(a)P binding to DNA and skin tumor initiating activity. In addition, EP was very effective in preventing the activation of the Ha-*ras* oncogene by DMBA. It should be noted that EP was much more effective against DMBA than B(a)P. Application of VP also inhibited B(a)P and DMBA covalent binding to DNA and skin tumor initiating activity in a dose-dependent manner, although higher doses of VP were required than doses of EP. Topical application of either EP or VP had no effect on the skin tumor initiating activity of a direct acting initiator such as N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG). The data suggest that the "A" moiety of EP and VP is needed to direct binding preferentially to the active sites of P450 isozymes involved in PAH activation and the "B" group of EP is also needed to function additionally as a mechanism-based irreversible inactivator. Although the suicide substrate EP is a very effective inhibitor of PAH carcinogenic activity, a combination of a PAH suicide substrate, an antioxidant, and a chemical to scavenge the electrophilic intermediate(s) of PAH(s), and a chemical to induce detoxification may be a rational and effective approach to a complete counteraction of the tumor initiation stage by PAH's.

The skin tumor promotion and progression stages are characterized by selective and sustained cell proliferation, differentiation alterations, and genetic instability leading to the specific expansion of the stem cells into papillomas and carcinomas. Because of their mechanism of action, a number of effective inhibitors of the skin tumor promotion and/or progression stages have been found. Antioxidants, free radical scavenging agents, anti-inflammatory agents, inhibitors of cell proliferation, and agents that counteract prostaglandin and polyamine synthesis are generally effective in counteracting these stages. We also have found that an N-acetyldehydroandine derivative AD-19, a potent free radical scavenging agent, was effective in preventing the formation of carcinomas during the tumor promotion and progression stages. AD-19 was also effective in suppressing TPA induced hyperplasia. We recently found that the ethylester of glutathione was more effective than glutathione in inhibiting both the skin tumor promotion and progression stages. Diethyl maleate, a chemical that reduces glutathione levels, was effective in enhancing progression. In addition overexpression of γ-glutamyltranspeptidase (GGT) which leads to a reduction in cellular glutathione levels also dramatically enhances progression.

In conclusion, a number of effective mechanism-based inhibitors have been found to counteract the initiation, promotion and/or progression stages of skin carcinogenesis. A rational and effective approach to counteracting the induction of cancer by environmental agents may well be the administering of a combination of agents that inhibit the different stages of carcinogenesis. The research was supported by Public Health Service grants CA-34962, CA-34521, CA-34890, and CA-43278 from the National Cancer Institute.

Transgenic approaches to the analysis of multistage carcinogenesis in the mouse.

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Tumour development in the mouse proceeds in a series of well defined stages involving sequential genetic alterations in oncogenes and tumour suppressor genes. Studies in mouse skin have shown that it is possible to derive cell lines which represent these different stages of carcinogenesis and which retain the appropriate genetic and biological characteristics of the *in vivo* tumours. We have developed a hybrid mouse system to study the genetic alterations in both primary tumours and cell lines using a panel of microsatellite polymorphisms distributed throughout the mouse genome (1). Both numerical and structural chromosomal changes are found which correlate with the transition of benign tumours to squamous carcinomas, or from squamous to spindle carcinomas. Benign tumours frequently have numerical alterations of chromosomes 7 and 6, whereas additional changes on chromosomes 11 and 4 are only seen in malignant tumours. The critical target genes on chromosomes 7 and 11 are the H-*ras* and p53 genes respectively. We have generated transgenic mice expressing *ras* genes in different sub-populations of cells within the epidermis in order to investigate the effects of compartmentalised expression of this oncogene. Previous results have shown that expression of a mutant human *ras* gene in suprabasal keratinocytes under the control of a keratin 10 promoter induced hyperkeratosis and papilloma formation (2). Expression of the same gene from a truncated basal keratin promoter (K5) induced acanthosis and formation of both keratoacanthomas and papillomas. In contrast to the results observed with the K10-*ras* transgenic mice, some of the benign tumours in K5-*ras* mice progressed to malignancy. Additional experiments have been carried out using animals in which the p53 gene has been inactivated by homologous recombination. The use of these transgenic and knockout animals has allowed us to demonstrate a causal role for H-*ras* and p53 in tumour initiation and tumour progression respectively (3). The cross-breeding of these and other transgenic or knockout animals should enable us to identify crucial roles played by a number of oncogenes, growth factors or tumour suppressor genes in

multistage carcinogenesis.

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II. Comparative Molecular Carcinogenesis

THE FUNCTIONAL ROLE OF THE TRANSCRIPTION FACTOR COMPLEX, AP-1, IN TUMOR PROMOTION AND PROGRESSION

G. Tim Bowden, John Levy and Rick Domann

Department of Radiation Oncology, University of Arizona Medical School, Tucson, Arizona, USA

The multistage process of mouse skin tumor carcinogenesis involves not only permanent gene alterations such as activation of oncogenes and inactivation of tumor suppressor genes by mutations but also transient alterations in gene expression mediated by tumor promoting agents. The classical tumor promoting agent 12-O-tetradecanoyl phorbol 13-acetate, TPA, can transiently induce the expression of genes in the mouse skin epidermis associated with hyperproliferation as well as some genes involved in tissue remodeling. TPA mediates its effects on the expression of genes through activation of the transcription factor complex called AP-1. AP-1 consists of a dimer of two Jun proteins or a heterodimer of a Jun and a Fos protein. The AP-1 complex is activated post-translationally to bind and transactivate a consensus enhancer element, TGAG/CTCA, through altered phosphorylation mediated by phorbol ester activation of protein kinase C. We have investigated the mediation of gene expression in mouse skin and in cultured keratinocytes by the non-phorbol ester tumor promoting agent, okadaic acid (OA). OA is a specific inhibitor of the phosphatases 1 and 2a and induces the expression of the same immediate-early and secondary response genes as TPA in mouse keratinocytes. We have found that OA induces p39 Jun and p55 Fos proteins to bind and activate transcription through a distinct non-TPA responsive enhancer element, TGACTCC (ORE), in mouse keratinocytes. This sequence has previously been described as a mutant TPA response element unable to bind to Fos and Jun. In the malignant keratinocyte tumor line PDV, Jun and Fos constitutively bind to and activate transcription through the ORE cis element. Our data support the idea that binding of Jun and/or Fos to the ORE cis enhancer element may be the result of activating a distinct gene regulatory pathway for AP-1. We have also obtained evidence that constitutive AP-1 activity plays a role in the maintenance of the malignant phenotype of SCC cell lines. A benign papilloma producing cell line called 308 was found not to express AP-1 DNA binding activity or transactivation from a TRE cis regulatory element. A malignant variant of the 308 cells, 308 10Gy5, was derived by ionizing radiation treatment. This malignant variant unlike its benign progenitor cell line constitutively expressed AP-1 DNA binding activity and transactivation from a TRE cis element. Furthermore, steady state transcript levels of the tumor associated AP-1 responsive genes, stromelysin, urokinase-type plasminogen activator, c-jun and c-fos were higher in malignant 308 10Gy5 cells than in benign 308 cells. To examine the role of this constitutive AP-1 activity in terms of the malignant phenotype, we stably expressed a transactivation deletion mutant of the human c-jun gene into the malignant cell line. This mutant c-jun protein has been shown to act in a dominant negative manner in

blocking AP-1 transactivation as well as transformation of cultured cells by activated ras and deregulated jun oncogenes. Expression of this dominant negative jun mutant protein in the 308 10Gy5 cells blocked transcriptional transactivation of AP-1 responsive reporter CAT constructs driven by jun, human collagenase and the mouse stromelysin promoters. These 308 10Gy5 cells expressing the dominant negative jun protein were also inhibited in their ability to form tumors in athymic mice. These results support the idea that inhibition of AP-1 mediated transcriptional transactivation is sufficient to suppress the malignant phenotype of SCC tumor cells. (This work was supported in part by USPHS grants CA-42230 and CA-40584 awarded to G.T.B.)

Antagonists of p21ras signalling.

J.L. Bos, B.M.Th. Burgering and J.P. Medema. Laboratory for Physiological Chemistry, University of Utrecht, The Netherlands.

p21ras is a small GTPase that functions as a switch in signal transduction. Thus far, most attention has been focused on the activation of p21ras signalling and it was found that a large variety of growth factors that bind and activate receptor tyrosine kinases use p21ras as a switch. Little is known, however, about processes that inactivate p21ras signalling. We have identified cAMP and calcium as antagonists of p21ras, .

In most fibroblasts, the cAMP analogue 8-bromo-cAMP negatively regulates growth factor-induced mitogenesis. We observed that 8-bromo-cAMP inhibits p21ras signalling. This inhibition was not observed at the level of the activation of p21ras but at the level of the raf1 kinase, a direct effector of p21ras.

We also measured the effect of both EGF and calcium treatment on activation of $p21^{ras}$ and ERK2 in human primary keratinocytes. We found that addition of EGF stimulated the activaty of ERK2. This stimulation was dependent on $p21^{ras}$ activity, since it was completely abolished by expression of a dominant negative mutant of $p21^{ras}$ ($p21^{ras(asn17)}$). Raising extracellular calcium (1.8mM) did not result in activation of ERK2. Surprisingly, calcium treatment inhibited EGF-induced stimulation of ERK2 activity.

In order to determine the site at which calcium treatment interferes in EGF-induced signaling we have analyzed the effect of calcium on the various steps that are involved in the EGF-induced, $p21^{ras}$-dependent, activation of ERK2. We observed that calcium treatment inhibited the EGF-induced $p21^{ras}$ activation. Calcium treatment, however, did not interfere with EGF-induced EGF receptor autophosphorylation as well as association of mSOS with the EGF receptor and Shc. This indicates that calcium treatment interferes with the direct activation of $p21^{ras}$. This novel type of cross-talk may play a role in the decision between proliferation and differentiation in human primary keratinocytes.

The role of Cyclin D1 in the progression of Chemically induced mouse skin tumors.

Claudio J. Conti
UT M.D. Anderson Cancer Center, Science Park-Research Division, P.O. Box 389, Smithville, Texas 78957

Over the last ten years, the research of my laboratory has been focused on the progression of chemically induced tumors in the mouse skin. Particularly, we have studied critical cytogenetic and molecular events in the conversion of papillomas into squamous cell carcinomas (1-4).

More recently we mapped the cyclin D1 gene (cyl 1) to the HBB-int 2 segment of chromosome 7 (5). This chromosome has been shown to be frequently trisomic in papillomas and carcinomas (6) and also is the target of mitotic recombination in the malignant tumors (7). We also have showed that cyclin D1 does not appear to be rearranged but it is amplified in a small number of tumors (5).

The expression of the cyl-1 gene is undetectable in Northern blots of normal or hyperplastic skin but it is detectable in early papillomas and markedly overexpressed in late papillomas and carcinomas (5). Immunoprecipitation analysis also showed the overexpression at the protein level (8).

We have recently developed an immunohistochemistry approach to detect the cyclin D1 gene expression in paraffin sections. Using this technique, we confirmed the absence of cyclin D1 overexpression in normal and hyperplastic skin but observed strong nuclear staining in the proliferative compartment of papillomas and carcinomas. A more detailed analysis of papillomas with this technique provided interesting results. Very early papillomas (< 0.5 mm at 8-10 weeks of promotion) are either negatives or present focal staining. In contrast, more advanced papillomas are positive for this technique. The staining is confined to the basal and suprabasal layers (proliferative compartment). Differentiated cells as well as cells from the stroma did not show any staining (8) In an effort to understand the possible role of cyclin D1 in skin carcinogenesis, we carried out a series of experiments to determine whether the overexpression of cyclin D 1 may be associated with the acquisition of autonomous growth (independence of the tumor promoter). Preliminary results from these experiments appear to be consistent with this hypothesis, i.e., tumors that grow after TPA withdrawal overexpress cyclin D1 at very early stages.

We have recently explored the possibility of a correlation between the overexpression of cyclin D1 and mutations of the H-ras gene. In collaboration with J. Filmus (Sunnybrook Health, Canada) we showed that activated ras induces significant overexpression of cyclin D1in rat intestinal cells (9). Therefore, we are presently studying the possibility that transformation properties of ras in epidermal keratinocytes are related to the overexpression and/or deregulation of cyclin D1.

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Similar abnormalities of the p53 and NF-2 genes in rodent and human tumors.

A.J.P. Klein-Szanto, S. Mitsunaga, S.Y. Zhang, E.R. Sauter, N. Kley and A. Bianchi

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p53 abnormalities have been detected in murine squamous cell carcinomas (SCC) of the skin induced by chemical carcinogenesis protocols. Approximately 50% of the tumors exhibited p53 mutations, most of which were G to T transversions when benzo(a)pyrene was used either as initiator or complete carcinogen. Interestingly very few papillomas or other premalignant lesion harbored p53 mutations, indicating that in these models, p53 abnormalities are related to conversion and/or tumor progression. In a study involving 16 pairs of primary SCC of the skin and their respective lymph node or lung metastases, no differences were observed between p53 positive and p53 negative tumors, suggesting that p53 mutations are not necessary for the acquisition of the metastatic phenotype.

p53 alterations were investigated in Human SCC of the head and neck region. This study yielded similar results ie, approximately 50% of tumors have p53 mutations, and no obvious association between metastasis and p53 could be established. Although SCC in both species showed a certain commonality with respect to the p53 changes, an obvious difference appeared when 201 human premalignant in situ lesions of the head and neck region were examined using immunohistochemistry with the p53 antiserum CM-1. This study showed that 40 to 50% of moderate to severe dysplastic lesions including carcinoma in situ, were p53 positive, thus indicating that p53 abnormalities appear earlier in human SCC tumorigenesis than in the rodent models.

The recently cloned NF-2 gene is a putative tumor suppressor gene that plays an important role in the development of acoustic schwannomas and other central nervous system tumors. We have recently shown that the NF2 gene also may play a role in an important group of histogenetically related tumors, ie. melanomas. Out of 20 human melanomas, six showed NF-2 abnormalities including point mutations, frameshift mutations and deletions. In a rodent model of skin melanoma using chemical carcinogens and UV light, similar molecular alterations were seen in 50% of the experimentally-induced melanomas.

The demonstration of similar molecular mechanisms of tumor development in rodent and humans reemphasizes the usefulness of murine models of human skin cancer.

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Characterisation of a ras-responsive element in keratinocytes

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Center for Nutrition and Toxicology, NOVUM, Karolinska Institute, S-141 57, Huddinge, Sweden.

The Long Terminal Repeat (LTR) of a retrovirus like 30s gene (VL30 B10) was used to define mechanisms of transcriptional regulation by an oncogenic c-Ha-ras in keratinocytes. Deletion mapping of the LTR and linking short sequences to a heterologous promotor made it possible to identify a 21 bp VL30 ras responsive elment (B10 RRE). The B10 RRE mediated both EGF- and ras- but not TPA-induced transcription in the Balb/MK keratinocyte celline.

Mutational analysis of B10 RRE indicated that cooperativity between several motifs is necessary for trans activation by an activated ras. Gel retartation studies showed that three specific DNA/protein-complexes were formed when using the B10 RRE as probe. When including antibodies recognizing transcription factors previously shown to be involved in ras-induced transcription one of these complexes was shown to contain proteins within the AP-1- and CREB-family of transcription factors. However, the formation of the other two complexes were unaffected by antibodies or competition with olgonucleotides corresponding to known binding sites. Interestingly, the B10 RRE did not mediate any ras-induced transcription when transfected into a fibroblast celline (NIH 3T3), indicating that the mechanisms behind the ras-induction observed in keratinocytes are celltype-specific.

p53-deficiency and v-ras^{Ha} cooperate in malignant conversion and altered growth regulation of murine epidermal keratinocytes. WC Weinberg, CG Azzoli, N Kadiwar and SH Yuspa, National Cancer Institute, Bethesda, MD, 20892, USA.

Epidermal carcinomas of mouse and man frequently contain mutations in the *p53* tumor suppressor gene. To clarify the contribution of this gene product to epidermal growth regulation, we have compared characteristics of keratinocytes from mice with and without a null mutation in the p53 gene, *in vivo* and *in vitro*. Epidermal keratinocytes were isolated from newborn mice following genotyping for p53 status by PCR analysis. Cultures were initiated by introduction of the v-*ras*^{Ha} oncogene via a retroviral vector. p53(+/+) and (+/-) cells expressing v-*ras*^{Ha} produced papillomas following grafting to nude mice, while p53(-/-) cells produced carcinomas observed as early as 3 weeks following grafting. The tumor growth rates varied depending on the p53 status of the grafted cells; after 3 weeks the average size of tumors derived from p53 (+/+), (+/-) and (-/-) keratinocytes expressing v-*ras*^{Ha} was 575 $\pm$ 155 (n=12), 1710 $\pm$ 303 (n=21) and 1643 $\pm$ 647 (n=7) mm^3, respectively. By 5 weeks, 10/19 p53 (+/-) and 0/8 p53(+/+) tumors contained foci of carcinoma cells. To explain the differential growth rates of these tumors the cell preparations were evaluated *in vitro*. Control cultures of each genotype responded to 1.2mM Ca^{2+} by 94-96% inhibition of DNA synthesis relative to cultures maintained in 0.05mM Ca^{2+}. Introduction of v-*ras*^{Ha} had no effect on Ca^{2+}-induced growth inhibition of p53(+/+) cells, but decreased the responsiveness of p53 (+/-) and (-/-) cells (97, 92.4, and 82.6% of control cells from each genotype, respectively, of parallel cultures in 0.05mM Ca^{2+}). p53(-/-)/v-*ras*^{Ha} cells also displayed decreased responsiveness to TGF-ß compared to cultures from p53(+/+) littermates (32% vs 68% suppression at 0.2ng/ml TGF-ß relative to control cultures). Thus, mutations in oncogenes (e.g. ras) and loss of tumor suppressor gene function (p53) cooperate in the malignant conversion of murine keratinocytes. This may be mediated, at least in part, by the decreased responsiveness to negative growth regulators observed in ras-initiated p53-deficient keratinocytes.

Role of receptor tyrosine kinases and their ligands in multistage carcinogenesis in mouse skin.

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Receptor tyrosine kinases (RTKs) are involved in growth control and alteration in several of these ligand/receptor systems may result in uncontrolled growth and oncogenic transformation. Current studies are designed to evaluate the role of several RTK signalling systems in multistage carcinogenesis in mouse skin. We have examined the ability of several classes of tumor promoting agents to modulate the expression of TGFα and its cognate receptor the epidermal growth factor receptor (EGFr) as well as signalling through the latter receptor. In addition, the levels (mRNA and protein) of TGFα and the EGFr have been examined in skin papillomas and squamous cell carcinomas (SCCs). Total RNA was isolated from SENCAR mouse epidermis treated with single applications of either TPA (3.4 nmol), chrysarobin (220 nmol), okadaic acid (OA) (2.5 nmol), or thapsigargin (8.5 nmol). Alternatively, total RNA was isolated from skin tumors generated by standard initiation-promotion regimens using DMBA as the initiator and either TPA or chrysarobin as promoters. Levels of TGFα transcripts were elevated in epidermis treated with all four promoting agents. Interestingly, both OA and thapsigargin led to rapid elevations in TGFα mRNA by 2-4 hrs. Elevated TGFα protein levels were detected in tumor promoter treated epidermis as well. In contrast, single applications of TPA did not dramatically alter levels of EGFr transcripts or protein. However, analysis of the EGFr from TPA treated skin revealed that this receptor had elevated phosphotyrosine levels as early as 3-6 hrs after TPA treatment indicating that this receptor becomes activated in promoter-treated skin. Studies are in progress to evaluate IGF-I/IGF-Ir transcripts in tumor promoter treated skin. Recently, two existing mouse mutations have been linked to the TGFα gene (Wa-1) and the EGFr gene (Wa-2). We have examined the responsiveness of Wa-1 mice to TPA-induced hyperplasia. Interestingly, Wa-1 mice were relatively resistant to TPA-induced hyperplasia. The reduced hyperplasia response correlated with reduced responsiveness to TPA-induced TGFα mRNA and protein production. Wa-2 mice are currently being examined.

Analysis of skin tumors revealed that most skin papillomas and SCCs exhibited elevated transcripts for TGFα, IGF-I, and EGFr, including early papillomas (10-13 weeks of promotion). We are currently examining IGF-Ir transcript levels in these same skin tumors. The present results suggest that induction of TGFα and signalling through the EGFr are important for tumor promoter-induced epidermal cell proliferation. Furthermore, constitutive elevations in the expression of TGFα, EGFr and IGF-I (and possibly IGF-Ir) may play an important role in the development of autonomous growth in skin papillomas and ultimately in the progression of these lesions. A long term goal of these studies will be to identify other critical RTKs and/or their ligands whose expression may be altered during multistage carcinogenesis in this model system and to identify mechanisms for all of these changes. Supported by USPHS grants CA57596 and CA37111 and ACS grant FRA-375.

III. In Vitro Studies and Cell Biology of Skin Neoplasia

Biochemical Pathways Relevant to the Development of Squamous Cancers of the Skin

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The introduction of the techniques of molecular biology as tools to study mouse skin carcinogenesis has provided more precise localization of biochemical pathways that regulate the tumor phenotype (1). This approach has identified genetic and epigenetic changes that are characteristic of each of the specific stages of skin cancer pathogenesis: initiation, exogenous promotion, premalignant progression and malignant conversion. The Harvey allele of the ras gene family has been identified as a frequent site for initiating mutations. Heterozygous activating mutations in c-ras^{Ha} are dominant, and effected keratinocytes hyperproliferate and are resistant to signals for terminal differentiation. An important pathway impacted by c-ras^{Ha} activation is the protein kinase C (PKC) pathway, a major regulator of keratinocyte differentiation. Increased activity of PKCα and suppression of PKCδ by tyrosine phosphorylation contribute to the phenotypic consequences of ras^{Ha} gene activation in keratinocytes. Tumor promoters disturb epidermal homeostasis and cause selective clonal expansion of initiated cells to produce multiple benign squamous papillomas. Resistance to differentiation and enhanced growth rate of initiated cells impart a growth advantage when the epidermis is exposed to promoters. The frequency of premalignant progression varies among papillomas, and subpopulations at high risk for progression have been identified. These high risk papillomas overexpress the α6β4 integrin and are deficient in TGFβ1 and β2 peptides. The loss of TGFβ1 directly contributes to premalignant progression since the introduction of the v-ras^{Ha} oncogene into epidermal cells derived from transgenic mice with a null mutation in the TGFβ1 gene produces initiated cells that have an accelerated rate of malignant progression when examined in vivo. Accelerated malignant progression is also found with v-ras^{Ha}-transduced keratinocytes from skin of mice with a null mutation in the p53 gene, and these cells are resistant to growth suppression induced by TGFβ. Malignant conversion is associated with a number of phenotypic alterations in benign tumor cells. Among these, the altered expression of a splice variant of the α6 integrin subunit is highly correlated to the invasive phenotype. The elucidation of biochemical pathways altered by genetic events which contribute to skin carcinogenesis is fundamental to the development of therapeutic approaches to prevent or eradicate epithelial cancers.

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Cellular and Genetic Control of Keratinocyte growth and differentiation.

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Addition of calcium to mouse primary keratinocyte cultures triggers a terminal differentiation program which closely resembles that of keratinocytes in the upper epidermal layers in vivo. Some of these changes - but not others - are also induced by treatment with the tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate). We have previously shown that tyrosine phosphorylation of a specific set of proteins occurs as a very early event in calcium- and TPA-induced differentiation and is required for these processes to occur. In particular, a single protein of ~80 kD becomes tyrosine phosphorylated at early times of TPA- and at later times of calcium-induced differentiation, while another protein of ~60 kD becomes tyrosine phosphorylated only at very early times of calcium exposure (within 5'). This latter protein was recently identified as a p62 protein which associates with the ras-GTPase activating protein (ras-GAP).

Nothing is known about the tyrosine kinases which are involved in induction of keratinocyte differentiation by calcium or TPA. We report here that a complex pattern of tyrosine kinase activation exists which is partially overlapping for calcium- and TPA-induced differentiation. In particular, exposure of keratinocytes to calcium, but not TPA, induces a very early (within 2') increase of a tyrosine kinase activity which immunoprecipitates with anti-phosphotyrosine antibodies and which may correspond to a novel ~60kD autophosphorylating kinase. In contrast to calcium, TPA induces very early (~2') activation of a kinase of the *src* family, c-*fyn*. The same c-*fyn* kinase is also activated by calcium exposure, but only at later times (8 hours). In parallel with the different time course of c-*fyn* activation, the p80-85 protein which becomes tyrosine phosphorylated in response to both calcium and TPA was identified with the *src*- and *fyn*-specific p80-85 substrate, an SH3-containing, actin binding protein which localizes at specific sites on the cell membrane. Thus, at least two distinct tyrosine kinase activities are induced at early times of keratinocyte differentiation, which may be responsible, among other things, of specific cytoskeletal/membrane changes associated with the differentiation process.

In a second set of studies, we are investigating the role of gap junctional intercellular communication (GJIC) in mouse keratinocyte differentiation. During calcium-induced differentiation, expression of α_1 (connexin 43) and β_2 (connexin 26) gap junction proteins are downmodulated, while the β_3 (connexin 31) and β_4 (connexin 31.1) proteins are induced. Concurrent with the switch in gap junction expression, transfer of fluorescent dyes, such as lucifer yellow or neurobiotin, is blocked in differentiating keratinocytes. In contrast, transfer of an amino acid or a nucleotide and electrical coupling proceed unimpeded or are actually increased after induction of differentiation. The possibility that selective gap junctional communication plays an important role in the syncronized control of keratinocyte growth/differentiation will be discussed.

ROLE OF CANCER GENE MUTATIONS AND ABERRANT INTERCELLULAR COMMUNICATION IN MULTISTAGE SKIN CARCINOGENESIS

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In order to elucidate molecular and cellular mechanisms of multistage carcinogenesis, we have been trying to identify presumptive "initiated" cells induced by genotoxic carcinogens and to examine how such cells are recruited into the promotion/progression stage of carcinogenesis. DMBA induces mouse skin papillomas, carcinomas and fibrosarcomas, as well as *in vitro* BALB/c 3T3 cell transformation, and the cells often contain A to T transversion mutations at the 61st codon of *ras* genes; papillomas and carcinomas contain Ha-*ras* mutation, while fibrosarcomas and transformed BALB/c 3T3 cells contain Ki-*ras* mutation. We have developed a highly sensitive method (PCR/RFLP) to detect A^{182} to T mutation in *ras* genes in cell cultures after DMBA exposure but before cell transformation. Our results clearly indicate that DMBA induces A^{182} to T mutation in both Ha- and Ki-*ras* genes. Since most of the DMBA-induced transformed cells contained Ki- but not Ha-*ras* genes, we concluded that only Ki-*ras* gene mutations contribute to transformation of BALB/c 3T3 cells. Our quantitative analysis suggests that about 25% of Ki-*ras* mutated (thus presumably "initiated") cells complete transformation and this can be increased by TPA treatment to >50%.

In humans, solar UV radiation plays a major role in the induction of skin cancers. UV-specific mutations (C to T, CC to TT) in the p53 gene have been observed in non-melanocytic skin cancers. We have recently developed allele-specific PCR and ligase chain reaction methods to detect CC to TT tandem mutations in the p53 gene in cultured human fibroblasts and keratinocytes after exposure to UV-B. Such mutations at codons 245 and 247/8 of the p53 gene (both of which have been found in human skin tumors) were detected in UV-exposed cells, but not in unexposed control cells. The analysis of normal skin biopsies from Australian skin cancer patients showed that cells from the shoulder, but not those from buttock, contain such UV-specific tandem mutations in the p53 gene, suggesting a direct role of UV in the induction of the p53 gene mutations which may subsequently contribute to skin carcinogenesis.

The above results from genotypic analysis indicate that the frequency of carcinogen-induced specific mutations in oncogenes and tumor-suppressor genes is high; 10^{-4} to 10^{-5} for *ras* A^{182} to T mutations induced in BALB/c 3T3 cells by DMBA and 10^{-6} to 10^{-7} for p53 CC to TT mutation in normal skin biopsies from sun-exposed sites. Thus only a small proportion of "initiated" cells seem to be recruited for further progression. As a possible mechanism by which this recruitment is determined, we have been examining the role of cell-cell interactions. Previous studies have shown that gap junctional intercellular communication (GJIC) ability is progressively decreased in mouse skin epidermal cells which show more malignant phenotypes. While the GJIC level in mouse epidermal cells was regulated by the extracellular Ca^{++} level, we have demonstrated that this effect is mediated by a Ca^{++}-dependent cell adhesion molecule, E-cadherin. Our preliminary results suggest that E-cadherin regulates the function of the gap junction protein, connexin 43, post-translationally in mouse epidermal cells. Down-regulation of GJIC by TPA is also mainly due to post-translational regulation of connexin 43 function.

Recent results from our own and other laboratories on connexin gene transfection indicate that these genes exert negative growth control in tumorigenic cells, supporting the hypothesis of a tumor-suppressive role for connexin genes. Other types of cell-cell communication certainly also play an important role in growth control. For example, we have recently demonstrated that in BALB/c 3T3 cells TGF-β suppresses transformed phenotypes only in the presence of non-transformed counterparts. These results are consistent with the idea that multistage carcinogenesis needs to be studied from the viewpoint that cancers arise from a disturbance of cellular society.

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Modulation of growth regulation and cell interaction of human skin keratinocytes during different stages of tumor progression

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With the spontaneously immortalized human skin keratinocyte cell line HaCaT and derived tumorigenic clones, we have established an in vitro system to study sequential genetic and phenotypic changes characterizing different stages of the multi-step process of skin carcinogenesis. Immortalization is considered an early stage in carcinogenesis and a prerequisite for in vitro transformation of human cells. Immortality of HaCaT cells is most probably caused by UV-induced mutations in the cell cycle control gene p53, which carries UV-type mutations in both alleles. In addition, the loss of potential senescence genes was essential for this transformation step as indicated by the loss of the short arms of chromosomes no. 4, 3, and 9, detected at earliest passages.

With further propagation, increasing chromosomal alterations occurred associated with improved growth autonomy of HaCaT cells in vitro. Nevertheless, the cells remained nontumorigenic after subcutaneous injection into nude mouse tested over more than 300 passages over a period of 6 years of in vitro propagation. Transfection of early passages with the cellular Harvey-ras oncogene (val-12; EJ) resulted in the formation of benign and malignant tumorigenic clones. The degree of transformation (malignancy) did not correlate with the level of ras oncogene expression nor the chromosomal integration site of the oncogene. Ras-oncogene-expressing tumorigenic clones had acquired increased growth autonomy in vitro (probably via an autocrine mechanism), while HaCaT cells still depended on serum- and mesenchyme-derived growth factors. Following subcutaneous injection into nude mice, immortal and benign cells formed keratinizing cysts which regressed or stayed constant over months. Malignant cells grew to progressively enlarging and invasive squamous cell carcinomas. This growth behavior of cells representing different transformation stages indicated a decreasing sensitivity to or increasing escape of mesenchymal growth control mechanisms.

We have developed an in vivo invasion assay where the early phases of tumor-host interaction can be studied while both compartments are separated by an acellular collagen matrix. Under these conditions, all HaCaT clones formed organized and differentiating stratified epithelia, but malignant cells rapidly elicited activation and directed migration of fibroblast and endothelial cells with angiogenesis into the collagen gel. Following rapid degradation of the matrix, carcinoma cells invaded the host mesenchyme and formed large tumor masses. This sequential development of invasion was paralleled by complex regulation mechanisms of the proliferative activity and of the expression of integrins, differentiation markers, and basement membrane components, as analyzed by immunohistochemistry and in situ hybridization.

These studies indicated that, with tumor progression, keratinocytes became increasingly independent of mesenchymal cell-derived growth factors (in vitro) and escaped of the negative growth control of the host environment. Thus, tumor progression in this keratinocyte cell system was correlated with genetic changes, altered cell phenotype, and, particularly with alterations in epithelial-mesenchymal interaction which favored cell growth in vivo and caused mesenchyme activation and angiogenesis preceding tumor invasion.

IV. Transgenic Mice

DEVELOPMENT OF TRANSGENIC MOUSE MODELS OF SKIN CARCINOGENESIS, D.R. Roop, A.M. Dominey, J.A. Rothnagel, D.S. Bundman, M.A. Longley, X.-J. Wang, D.A. Greenhalgh, Baylor College of Medicine, Department of Cell Biology, One Baylor Plaza, Houston, TX 77030

The ability to stably introduce genes into the germline of mice has greatly enhanced prospects for the generation of animal models of human diseases. The need for such animal models is becoming increasingly apparent as novel pharmaceuticals are developed which are specifically designed to inhibit expression of human viruses or counteract the effect of mutated genes that occur in human cancers. Current efficacy assessments of these new therapeutic agents are restricted to in vitro models which do not allow evaluation of delivery routes nor assessment of other factors known to affect disease processes in vivo. In addition, the prospects for utilizing gene therapy to treat cancer are coming closer to reality. Therefore, animal models of human cancers would be useful to assess the therapeutic potential of these approaches. We have recently developed a vector which specifically targets gene expression to the epidermis of transgenic mice. The epidermis is an ideal tissue for the development animal models since it serves as a general model for epithelial cancers and its accessibility allows macroscopic observation of neoplastic events and easy assessment of therapeutic potential. Using this vector, we have targeted the expression of several genes which have been implicated in epithelial carcinogenesis (ras^H, fos, TGFα and the E6/E7 genes of HPV18). Our ability to target gene expression to the epidermis and generate animals expressing single or multiple oncogenes has produced an easily accessible, transgenic mouse model which appears unique among similar models in that it closely mimics the discrete stages involved in human carcinogenesis. For instance, our mice develop pre-neoplastic hyperplasia and regression prone papillomas when expressing a single oncogene, autonomous papillomas with double oncogenes and exhibit a very low frequency of spontaneous malignant conversion (occurring only in old (>16 months) mice). To determine if loss of a tumor suppressor gene will accelerate malignant conversion, we are currently mating these mice with transgenic mice that are null (as the result of a knock-out experiment) for the tumor suppressor gene, p53.

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FORCED EXPRESSION OF KERATIN K10 INHIBITS CELL PROLIFERATION.
EXPRESSION OF A DOMINANT NEGATIVE FORM OF EGF-R IN THE SKIN OF TRANSGENIC MICE.

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Keratin K10 is expressed in suprabasal epidermal cells. These cells are assumed to be postmitotic and normally they do not divide. In situations such as tumors, where suprabasal cells are under active proliferation, the expression of this protein is reduced or even absent. To study the possible involvement of keratin K10 in the control of cell proliferation, we transfected several epithelial and non-epithelial established cell lines with different plasmid constructs coding for keratin K10. We also used primary cultures of mouse keratinocytes derived from the epidermis of transgenic mice in which human keratin K10 expression is driven by the bovine keratin K6 promoter. In all the cases the forced expression of the keratin K10 resulted in a considerable decrease in the number of the cells able to enter into S-phase, as visualized by bromodeoxyuridine (BUdR) incorporation.

These results suggest that K10 could play an active role in the growth arrest of suprabasal epidermal cells.

To study the role of EGF/TGF-α in skin development, function and pathology, we have generated transgenic mice overexpressing a truncated form of the EGF-R gene (lacking the citoplasmic domain) under the control of the keratin K5 promoter. As expected, in skin the expression of the transgene occurred in the epidermal basal layer as well as in the outer root sheath of the hair follicles. The transgenic mice, which are viable, showed a phenotype of wavy hairs and wiskers with variable intensity. Some animals were similar to waved-1 and waved-2 mice and also to the null TGF-α mice recently described. Others, with an apparently stronger phenotype, developed very short hairs which became atrophic and scarce with age. Thus, our approach seems to be able to interfere with the EGF-R signal transduction system. These mice will be useful in wound healing and carcinogenesis studies.

TARGETED EXPRESSION OF A MUTANT H-RAS GENE TO THE HAIR FOLLICLE PRODUCES BEGIGN TUMORS WHICH UNDERGO FREQUENT MALIGNANT PROGRESSION.

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As part of our studies on mouse skin carcinogenesis, we have generated transgenic mice which spontaneously develop squamous carcinomas. The promoter element from the bovine equivalent of the human cytokeratin 5 gene was used to target the expression of a mutant Ha-ras transgene to the basal cells of the skin in mice. Expression was highest in the basal cells of the outer root sheath of the hair follicles, as determined by using a lacZ marker gene in transgenic mice. Using the keratin 5 ras construct, a number of founder animals were produced which showed marked hyperplasia of the entire epidermis at birth. This was associated with a decrease in both dermal and adipose layers of the skin. In addition, extensive raised hypopigmented areas were visible on the animals, which were composed of acanthotic downgrowths, which rapidly become dysplastic and progress to carcinoma in situ and invasive carcinoma. This phenotype was lethal, with pups dying by day 10 post partum. A milder phenotype was noted in five transgenic lines in which mice show no evidence of hyperplasia but develop spontaneous tumours. In addition to the normal papillomas, two other histological types of tumour were observed. These could be classified as either acanthotic growths or keratoacanthomas, and appeared to arise from hair follicles. The keratoacanthomas, in particular, progress to squamous carcinomas with moderately high frequency. Thus, targeting mutant ras expression to the basal layer results in benign tumours which can progress to fully malignant carcinomas.

Interestingly, tumour development in the majority of transgenic lines does not appear to be promoted by TPA or wounding stimulus.

A GENETIC TEST OF THE ROLE OF c-FOS IN SKIN TUMORIGENESIS

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Advances in the field of mammalian genetics have provided novel approaches to examine well-established models of *in vivo* carcinogenesis. The development of transgenic and "knockout" mice has allowed us to explore the relevance of particular genes in the neoplastic process. In the mouse skin model of carcinogenesis, these techniques have served to confirm previous notions associating specific genes to the various steps of skin tumor formation. Mutations in the *c-H-ras* gene have been shown to be important for initiation, while loss of the wild-type *p53* gene has been linked with progression to malignancy. Utilizing these approaches, several studies have used transgenic mice overexpressing either *c-fos* or *v-fos* in their skin to suggest a role for this proto-oncogene in either promotion or progression of epidermal tumors. Taking advantage of mice lacking *c-fos* generated by our lab, we decided to ask whether skin tumor development is possible or different in the absence of *c-fos*.

In order to be able to analyze mechanistic issues without the complication of the possible introduction of new mutations, we decided to avoid the use of initiators in our study. Instead, we chose to mate our Fos-less mice with a transgenic strain expressing an activated *v-H-ras* gene in its skin. Upon treatment with tumor promoters, this transgenic strain quickly develops papillomas. Over one-hundred mice derived from these crosses were painted with TPA according to standard promotion protocols. Treatment was completed in late December.

To date, papillomas are visible in mice of all genotypes, including those expressing the *v-H-ras* transgene in a Fos-less background. Furthermore, the time to first appearance of tumors, and the number of papillomas per mouse is very similar in all treated mice. The gross morphology of the papillomas found in Fos-less mice was initially identical to that of wild-type mice. Lately, the Fos-less papillomas seem to have become less aggressive; many of them have little vascularization and some are clearly regressing. A detailed histological analysis of these differences is currently in progress.

In a separate study, our laboratory has shown that fibroblast cells derived from Fos-less mice are completely unable to induce the expression of several metalloproteases in response to stimulation by growth factors. Since these proteins have been implicated in the progression of benign tumors towards malignancy and invasion, this result suggests that the papillomas present in our treated Fos-less mice may not be able to progress into carcinomas, or that they may do so at a reduced rate. Hence, we are carefully monitoring the behavior of the Fos-less papillomas to see whether *c-fos* is required for tumor progression *in vivo*.

Our data thus far seem to indicate that *c-fos* is not required for promotion of skin tumors. In addition, we are also able to conclude that lack of Fos does not act as an initiating agent, for Fos-less mice that did not carry the *v-H-ras* transgene did not develop papillomas upon TPA treatment. When integrated with our *in vitro* results these findings raise the interesting possibility that *c-fos* may act as an oncogene not by increasing the rate of cell proliferation, but rather, by controlling the expression of certain genes which may be critical for tumor progression.

ALLELOTYPING AND DELETION MAPPING OF CHROMOSOME LOSSES IN BASAL CELL CARCINOMAS USING MICROSATELLITE POLYMORPHISMS.

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The detection of frequent loss of heterozygosity (LOH) at specific loci has been used to identify chromosome regions that contain putative tumour suppressor genes. The mapping of the naevoid basal cell carcinoma syndrome (NBCCS) to 9q22-31 and the high frequency of 9q allele loss in sporadic BCCs indicates that inactivation of one or more tumour suppressor genes on 9q is important in BCC development. In order to relate 9q changes to other genetic alterations in sporadic BCCs we have determined the pattern and extent of allele loss in a series of 45 BCCs using polymorphic microsatellite markers from all 39 autosomal chromosome arms. LOH of one or more 9q alleles was observed in 28 of 45 informative BCCs. 10 of the 28 BCCs with 9q LOH showed additional losses on one or more chromosome arms. Three tumours showed loss of other regions with retention of heterozygosity of 9q markers. LOH of 1q was seen in 6 of 46 informative BCCs and 3p in 3 of 39 tumours. Interestingly LOH of chromosome 17p which is a common event in many internal malignancies was not seen in any of the BCCs examined. Deletion mapping using a large number of microsatellite markers mapped to 9q identified several tumours with interstitial deletions. Our results indicate that (1) the pattern of chromosome 9 loss in BCCs and SCCs is different and (2) allelotyping reveals multiple differences in chromsome loss between BCCs and SCCs. Preliminary data suggests that many of these changes are present at the pre-invasive stage.

V. Cell Differentiation and Skin Carcinogenesis

Regulation of expression and functional activity of cadherins in skin carcinogenesis.

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Cadherins are calcium-dependent cell adhesion molecules involved in acquisition and maintenance of tissue architecture and homoeostasis. Cadherin activity can be modulated at the level of gene expression or by influencing their functional adhesive activity. Misregulation of E-cadherin (E-CD) at either level have been reported to occur in a variety of epithelial carcinomas, whereas less attention has been paid to other members of the family. Skin carcinogenesis provides an excellent model to study the role of two members of the cadherin family in tumor development and progression: P-cadherin (P-CD) restricted to the basal proliferative layer and E-CD expressed in the basal and living suprabasal layers of adult epidermis. During mouse skin carcinogenesis downregulation of E-CD occurs during progression of squamous cell carcinomas, whereas both E- and P-CD are completely switched off in fully undifferentiated spindle cell carcinomas. Our previous studies showed an important role for E-CD in tumor progression as reexpression of E-CD was able to partly suppress the tumorigenic properties of squamous carcinoma deficient cells. However, the expression of either E- or P-CD was not sufficient to modify the fibroblastic phenotype and the tumorigenic properties of spindle carcinoma cells indicating the absence of addtional factor(s) required for cadherin activity.

The functional activity of cadherins is believed to be dependent of their interaction with the cytoskeleton which is mediated by the association of the cytoplasmic domain with at least three cytoplasmic proteins: α–, β– and γ–catenin. Immunoprecipitation analysis of normal keratinocytes and squamous carcinoma derived cells showed the association of cadherin molecules with the three catenin components. However, similar analysis on E- or P-CD transfectant spindle cells showed that only α– and β–catenin are associated to the exogenous cadherins. The lack of association of γ–catenin to the cadherins is correlated with the absence of plakoglobin expression in parental and transfectant spindle cells. These results support that γ–catenin corresponds to the plakoglobin component and suggest a role for plakoglobin in the functional activity of cadherins. This hypothesis is being tested by stable transfection experiments on parental spindle cells and one E-CD+ derivative with a vector containing the whole coding sequence of plakoglobin cDNA and a hygromicin resistance vector. Several clones expressing plakoglobin have been recently isolated and are presently being analysed for their morphophenotype and tumorigenic behavior.

To further understand the genetic mechanisms involved in the downregulation of E- and P-cadherin at different stages of mouse skin tumor progression the 5' upstream sequences of the mouse P-CD gene have been isolated and analysed for promoter activity. Transient transfection assays of various P-CD constructs containing different potential regulatory sequences and a luciferase reporter gene have been carried out in expressing and non-expressing cells. The results obtained indicate that the basal activity of the P-CD promoter in mouse keratinocytes is driven by a combination of a CAAT box (-65), a GC-region (-88) and a putative AP2-binding site (-101). Interestingly, the different P-CD promoter constructs are basically inactive in spindle carcinoma cells as well as in normal fibroblasts. Similar studies with various E-CD promoter constructs (provided by J. Behrens and W. Birchmeier) indicate that the expression of E-CD gene in expressing mouse keratinocytes is driven by a combination of an E-pal element (-75 to -86), a CAAT box (-65) and a GC-rich sequence (-58). The different E-CD promoter constructs are inactive in spindle carcinoma cells and showed reduced levels of expression in E-CD deficient squamous carcinoma cells. These results indicate the absence or inactivation of nuclear factors involved in regulation of both cadherin genes during development of spindle cell carcinomas.

KERATIN MUTATIONS IN SKIN DISEASES

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Keratins are a large family of stuctural proteins expressed by epithelial cells at different locations in the body. Over thirty different genes have been identified in the human genome, in two subfamilies (type I and type II) which are coexpressed in type I-type II pairs, depending on, or maybe even determining, the state of differentiation of the cells and their functional capacities (Lane, 1993). Monoclonal antibodies which can distinguish between these different keratin proteins have therefore established a place for themselves in the battery of tools used in diagnostic pathology, as valuable markers of differentiation and therefore tissue type markers (Lane and Alexander, 1990).

It is widely accepted that intermediate filament expression in tumours appears to match that of the tissue of origin. However, extensive immunohistochemical analysis of human tissues in development, differentiation, wound healing and cancer, has revealed that keratin expression can alter in response to the surroundings of the epithelial cells. Whilst this may limit the value of anti-keratin antibodies for tracing cell lineages, it also increases the possibility of interpreting certain changes in keratin expression in a diagnostically significant context. For example, a shift in keratins is often observed in epidermal squamous cell carcinomas which have become invasive, from the normal expression of keratinocyte keratins to expression of simple epithelial keratins. Without an understanding of the role of keratins in epithelial sheet tissues, it is difficult to predict what the biological significance could be of such a shift in keratin expression.

However, recent discoveries of pathogenic keratin mutations (by our lab and others) have made the purpose and function of these structural proteins much clearer. In a group of hereditary skin blistering diseases including epidermolysis bullosa simplex, the epidermal keratinocytes fracture and break on physical stress. This is because these cells no longer have an effective cytoskeleton - the intracytoplasmic network of keratins is either faulty, reduced or missing. The discovery that mutations in keratin genes are responsible for a range of pathological conditions involving fragility of epidermis has provided important information on the function of intermediate filaments in tissues: it has provided a dramatic demonstration of how keratins function as a cytoskeleton in the true sense, by defining the resilience and plasticity of cells. The resilience of a cell's cytoplasm permits or restricts certain types of cellular functions, and this could be the basis for the great heterogeneity of expression characteristic of the intermediate filament gene family.

There are four clinical types of epidermal fragility syndromes that have now been shown to be associated with keratin defects: epidermolysis bullosa simplex (defects in K5 and K14 leading to basal keratinocyte fragility (Lane et al., 1992)), bullous ichthyosiform erythroderma (mutations in K1 and K10 leading to lysis in suprabasal keratinocytes (McLean et al., 1994)), epidermolytic palmo-plantar keratoderma (mutations in K9 leading to defects in palmo-plantar epidermis), and

most recently another variant, ichthyosis bullosa of Siemens, which shows very superficial splitting and in which we have now identified mutations in K2e.

We have now identified 10 mutations in 14 families with various types of epidermolytic ichthyoses. These mutations are not distributed randomly, but fall into a number of clusters along the protein sequence. There is an indication that the severeity of the diseases may be associated with the cluster in which a mutation falls, with the most severe diseases associating with mutations in the conserved motifs at the two ends of the overall rod domain. All the keratin mutations identified to date occur in up to 6 clusters along the consensus protein structure (Rugg et al., 1993), i.e.: (1) H1 domain (type II keratins only), (2) helix initiation domain in 1A (severe phenotypes; most common site), (3) second half of 1A (mild phenotypes), (4) L12 linker domain (mild phenotypes), (5) second half of helix 2B (mild phenotypes) and (6) the end of 2B, the helix termination domain (severe phenotypes). This clustering will tell us more about the role of different sites along the rod domain in the process of filament assembly.

The faults in the cytoskeleton which lead to cell fragility are clearly linked to point mutations at strategic locations along the keratins, creating critical weaknesses at points of high structural conservation. Identification of sites of mutations helps us to understand the packing and assembly of intermediate filaments like keratins. Information from these skin diseases about the importance of precise sequence specification for correct function for intermediate filaments may tie up with explanations of how different keratins equip an epithelial cell for different sorts of behaviour, and may shed light of the selection pressure underlying shifts in keratin expression observed in carcinomas.

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Alterations of epithelial differentiation associated to late stages of mouse skin carcinogenesis

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In mouse skin carcinogenesis, the development of malignant carcinomas is linked to gross alterations of epidermal differentiation. Characteristic changes in cell adhesion and cytoskeletal proteins have been described associated with the invasive phenotype. For example, loss or downregulation of the cell-cell adhesion protein E-cadherin (E-CD) (1, 2) and the aberrant expression of the embryonic/simple epithelial keratin K8 (3, 4) are found frequently during progression in vitro and in vivo. We are investigating the implication of these changes in malignancy.

In a study we have analyzed the metastatic ability of the HMSV-induced carcinoma cell line HaCa4, which is E-CD (-) and K8 (+), and that of cell clones derived by transfection with the E-CD gene. Clones E-CD (+) were, in general, as metastatic as the parental cell line and expressed high levels of the viral ras oncogene. However, the clone E24, E-CD (+) and K8 (-), which showed reduced levels of viral ras oncogene expression, lost completely the capacity to metastasize although remained tumorigenic. These results do not support the view of the E-CD gene as a suppressor of metastasis and suggest a correlation between the dosage of ras oncogene expression and metastatic ability in mouse epidermal carcinogenesis.

On the other hand, we have studied the effects of TGF-β in the differentiation characteristics of cultured mouse epidermal keratinocytes. Chronical exposure to TGF-β of a chemically-transformed epidermal cell line (PDV) induced cell dispersion, membrane ruffling and increased local motility. These changes were accompannied by a partial inhibition of cell growth. Long-term treatment with the factor led to a drastic conversion to a fibroblast-like morphology that correlated with changes in the expression of epithelial and mesenchymal differentiation protein markers: downregulation of E-CD and keratins and upregulation of vimentin. Cells reverted to the epithelial phenotype when TGF-β was withdrawn from the culture medium. No significant differences in tumorigenicity were found between treated and untreated cells, although histological examination of the tumors showed a correlation between the morphology of injected cells and the differentiation grade of induced tumors. When immortalized, nontumorigenic, epidermal cells (MCA3D) were treated in the same manner, no drastic phenotypic changes were observed, proliferation was blocked and cells finally died. The relevance of these results for epidermal carcinogenesis is discussed.

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ABERRANT SYNTHESIS OF KERATIN K13 IN MURINE EPIDERMAL TUMORS: REGULATION OF EXPRESSION BY CA^{2+} AND RETINOIC ACID.

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Alterations in keratin expression in DMBA/TPA induced epidermal tumors of mouse skin are characterized by the gradual down-regulation of the expression of the epidermal differentiation specific keratins K1 and K10 in papillomas and loss of expression of these keratins in carcinomas. Concomitantly, these tumors exhibit an increasing tendency to aberrantly express the type I keratin K13 which normally constitutes - together with K4 - the differentiation specific keratin pair of internal stratified epithelia (1). The expression characteristics of K13 in papillomas and carcinomas qualify this keratin as a positive marker for malignant progression during two-stage carcinogenesis of mouse skin. There is evidence for a causal relationship between the activation of the Ha-ras oncogene and the aberrant K13 expression in DMBA/TPA induced tumors, but investigations in UVB-induced murine epidermal neoplasias suggest also alternative mechanisms (2, 3).

Aberrant synthesis of K13 is also a feature of papilloma- or carcinoma-derived cell lines in which the expression of the keratin is controlled by the concentration of Ca^{2+} or retinoic acid (RA) in the culture medium (4). Since these two mediators of epithelial differentiation are also involved in the regulation of K13-expression in cultured primary keratinocytes of internal epithelia, the transformed epidermal cell lines represent ideal systems to study the molecular mechanisms of Ca^{2+}- or RA-controlled K13 gene expression *in vitro*.

Transfection experiments with reporter gene constructs of K13 gene 5'sequences (up to 6.5kb) did not reveal the presence of Ca^{2+}- or RA-responsive enhancer elements in this part of the K13 gene. A short sequence element in the proximal promotor of the K13 gene with high homology to the RA-responsive element (RARE) of the nuclear RA-receptor β_2 gene turned out to be transcriptionally silent due to two A-T base changes in the second half motif of the wild type element. Sequencing of PCR amplified genomic DNA from papillomas and carcinomas ruled out the possibility that K13 may become aberrantly inducible by RA through the restoration of a partially or fully functional K13 RARE generated during tumor initiation with the mutagenic carcinogen DMBA. In contrast, both Ca^{2+}- and RA-responsive elements could be identified in the 3'-noncoding region of the K13 gene. There is evidence that Ca^{2+}-regulated enhancer sequences are restricted to a 0.5kb fragment 3.9kb downstream the stop codon. The fragment contains two AP1 sites. AP1 containing 3'-sequences have also been described as Ca^{2+}-mediated enhancers of type II keratin K1 gene expression (5, 6) thus indicating a common mechanism for the regulation of expression of differentiation specific keratin genes in stratified squamous epithelia. The RA-responsive region of the K13 gene is located within a 0.5kb fragment that is contiguous with the Ca^{2+}-sensitive fragment but lacks a

classic RARE. Whereas the Ca^{2+}-enhancer element is active only in keratinocytes, the RA-regulated element is also active in fibroblasts. Ongoing experiments are aimed at further characterizing the respective enhancer elements and at identifying their corresponding transcription factors.

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VI. Human Skin Carcinogenesis

Sunlight, skin cancer, and p53

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To learn how sunlight leads to human skin cancer, we first sought to identify a gene that is mutated by UV light in human skin tumors. We found that 92% of invasive squamous cell carcinomas of the skin (SCC) and basal cell carcinomas (BCC) contained aberrations of the p53 tumor suppressor gene or protein. 91% of SCC from New England contained mutations in the p53 gene, each altering the amino acid sequence (reference 1 and subsequent data). 56% of BCC carried point mutations; several had two mutations, one in each allele (2).

Involvement of UV light in these p53 mutations was indicated by the distinctive mutational signature of UV: i) the presence of several CC→ TT double-base changes and ii) a UV-like occurrence of mutations exclusively at sites of adjacent pyrimidines, including a high frequency of C→ T substitutions. p53 mutations in internal malignancies do not show these UV-specific mutations. The clarity of this result stems from the fact that a tumor suppressor gene leads to cancer by being inactivated; since many base substitutions can inactivate a gene, the mutations originally made by the carcinogen will be well-represented in the phenotype. In contrast, oncogenes require a gain-of-function, imposing a severe phenotypic selection.

Combining mutation data from SCC and BCC, we found the converse evidence for a role of UV mutagenic photoproducts: mutations were absent where UV photoproducts should not form. Although mutations were found at several of the codons that are mutation hotspots in internal malignancies (3), none were found at hotspot codons 175 or 273. These do not have adjacent pyrimidines, and so should not form UV photoproducts.

The prevalence of mutations at the C of a dipyrimidine site implies that the oncogenic photoproducts are dipyrimidine photoproducts containing cytosine. These include the TC, CT, and CC cyclobutane pyrimidine dimers, the TC and CC (6-4) photoproducts, and the Dewar isomers of the (6-4) photoproducts (4). In nine of the skin tumors, it is likely that the carcinogenic photoproduct was a CC cyclobutane pyrimidine dimer. This is because these mutations occurred at sites of 5-methylcytosine, which is known to block the formation of (6-4) photoproducts but not cyclobutane dimers (5).

In BCC, the existence of mutation hotspots altered the process of inactivating p53 compared to other cancers: allelic loss was rare, but 45% of the point mutations were accompanied by a second point mutation on the other allele. At least one of each pair was located at a mutation hotspot (2). Sunlight, acting at mutation hotspots, appears to cause mutations so frequently that it is often responsible for two genetic events in BCC development.

Actinic keratoses (AK) of the skin are precancerous lesions that have the potential to progress to squamous cell carcinoma. In 45 AK's obtained from New England patients, 58% carried a mutation in the p53 gene. Patients with multiple AK's had different mutations in each AK. Most mutations were again UV-like. Their uniform presence throughout the lesion suggests that they preceded the clonal expansion of the AK. AK's therefore appear to be clones arising from independent sunlight-related p53 mutations. p53 mutations are thus early events in the development of SCC.

What function does p53 perform in normal keratinocytes, and why does abrogation of this function lead a keratinocyte down the path to skin cancer? To address this question, we examined p53 knockout mice (from T. Jacks, MIT) for sunburn cells, apoptotic keratinocytes induced by over-exposure to UV radiation. Sunburn cells were rare in p53 -/- mice, and were found at intermediate levels in -/+. This result suggests that a sunburn is a p53-dependent mechanism for killing sun-damaged keratinocytes. A prior p53 mutation, however, could permit a sun-damaged cell to survive and clonally expand as an actinic keratosis, leading to skin cancer.

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PAPILLOMAVIRUS AND SKIN CANCER

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Rabbit skin papillomas and derived carcinomas induced by the cottontail rabbit Shope papillomavirus (CRPV) provided the first model of viral carcinogenesis in mammals. This led to such concepts as virus masking in tumors and synergy between viruses and chemical carcinogens in the development of cancers. The first evidence for the role of human papillomaviruses (HPVs) in human cancer was the association of a specific HPV type (HPV5) with skin cancers of epidermodysplasia verruciformis (EV), a rare genetic disease. Other specific HPV types (HPV16, HPV18,...) play a central role in the development of carcinoma of the uterine cervix, the second most common cancer in women world-wide.

Papillomaviruses are small epitheliotropic DNA viruses. Their genome, a double-stranded covalently closed DNA molecule of about 8000 base pairs, comprises the origin of replication and transcriptional regulatory elements, upstream the coding sequences located on the same DNA strand. The virus-encoded proteins are involved in viral DNA replication and transcription regulation (E1,E2), immortalization and transformation (E5,E6,E7), interaction with keratins (E4), or represent structural capsid proteins (L1,L2).

Of the 73 HPV genotypes characterized so far, about half infect specifically the epidermis and half the squamous epithelium of mucous membranes (most often genital). A number of papillomaviruses induce benign, self-limiting papillomas (warts, condylomata acuminata) which usually regress spontaneously or after treatment. Other HPV types cause premalignant lesions such as cervical intraepithelial neoplasia, and some of them are associated with the development of malignant tumors (skin EV cancers and genital carcinomas).

HPVs replicate exclusively in terminally differentiating keratinocytes of the benign and premalignant lesions they induce. No cell culture system allows HPV propagation after in vitro infection or transfection. Genital oncogenic HPV types are capable of immortalizing human keratinocytes in vitro but this involves integration of viral DNA sequences, an event which does not occur normally in the life cycle of HPVs. Little is known on the mechanisms of the formation of a papilloma but it is likely to involve the interaction of E6 and E7 proteins with cellular proteins controlling the cell cycle, such as p53 and pRB, and that of E5 protein with growth factor receptors.

EV is a lifelong disease, characterized by disseminated cutaneous flat wart-like lesions and macules. Invasive carcinomas develop from benign lesions through intraepithelial neoplasia in about half of the patients, usually on light-exposed areas, about 20 years after the onset of the disease. Most patients with EV have an impaired cell-mediated immunity. EV can be defined as a genetically conditioned abnormal susceptibility to infection by a group of specific related HPV types and to the oncogenic potential of some of them. At least 20 specific HPV types have been characterized so far in benign EV lesions. The genome

of HPV5 and occasionally of HPV5-related types (HPV8,14,17,20) are detected in EV premalignant lesions and cancers. These HPVs are harmless for the general population and their reservoirs have not been identified. EV tumor progression does not involve either integration of viral sequences to the cell genome or point mutations affecting the upstream regulatory region and the E6 and E7 genes. High levels of E6 and E7 transcripts are found in EV cancers, supporting that the expression of E6 and E7 is required at all stages of tumor progression. The cellular target proteins of HPV5 E6 and E7 proteins and the alterations of the cell genome involved in tumor progression have not yet been identified. EV HPV infection of keratinocytes leads to the induction of cytokines (TNFα and TGFβ), which may interfere with local immune responses. EV is the human counterpart of the rabbit Shope papillomas and carcinomas. Recent data indicate that CRPV E6 protein is the target involved in the systemic wart regression observed in a proportion of rabbits and that wart regression and cancer development in persistor rabbits are controlled by genes in the class II region of the major histocompatibility complex.

Data available do not point to a major role of EV HPVs and other known HPVs in skin carcinogenesis in the general population. This stresses the importance of the genetic factors in EV HPV infection, affecting either an intracellular control of EV HPV gene expression or specific immune responses to virus-encoded tumor antigens, as suggested by preliminary data pointing to a significant association of EV with a specific HLA DR-DQ haplotype.

Skin carcinogenesis in EV and progression of persisting cervical intraepithelial neoplasia into invasive carcinoma share many features : (i) infection with specific HPV types, (ii) continuous expression of viral E6 and E7 oncoproteins which, in the case of HPV16- and 18-associated carcinogenesis, results in the inactivation of p53 (E6) and pRB (E7) tumor suppressor proteins, and (iii) the requirement for tumor progression of cell genome alterations, still poorly understood, resulting from the chromosomal instability induced by viral gene expression or from mutagenic events.

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EGF-R LOSS OF FUNCTION IN EPIDERMIS OF TRANSGENIC MICE

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EGF/TGFα is a potent mitogen for a variety of cells through its binding to the EGF receptor (EGFR). In addition TGFα is thought to be an important autocrine factor affecting growth of epidermal cells. Overexpression of TGFα in the basal layer of the epidermis of transgenic mice facilitates papilloma formation upon wounding or TPA treatment without the need for a carcinogen as initiator (Vassar et al, Mol. Cell Biol. 12:4643-4653, 1992).
We have generated transgenic mice overexpressing a truncated form of the EGFR gene (lacking the citoplasmic domain) under the control of the keratin K5 promoter. As expected, the expression of the transgene occurred in the basal layer of estratified epithelia. Mice, which are healthy and fertile, showed a phenotype of wavy hair and curly whiskers. Some of them show open eyelids at birth. This phenotype is remarkably similar to the mutant waved-1 and waved-2 mice and also to the null TGF-α mice recently described (Mann et al. Cell 73: 249-261, 1993). Thus, signal transduction throuh the EGFR seemed to be either abolished or greatly reduced in our transgenic mice. We are currently performing skin carcinogenesis experiments on our loss of function EGFR transgenic mice to determine whether tumor development is inhibited .

ANALYSIS OF P AND E CADHERIN PROMOTERS IN MOUSE EPIDERMAL KERATINOCYTES

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The anti-invasive role of the cell adhesion molecule E-cadherin has been several times reported. Our previous data on the expression of E-cadherin (E-CD) in mouse skin carcinogenesis showed a clear inverse correlation between the amount of E-CD and the tumorigenicity of different cell lines (Navarro et al., J.Cell Biol.,115:517, 1991). Although this correlation was not found for the P-cadherin (P-CD), both cadherins are down-regulated in spindle cell carcinomas (CarB and CarC), suggesting that both cell adhesion molecules can be implicated in the final stage of epidermal carcinogenesis as well as in the maintenance of cell shape (Navarro et al, J. Cell Science, 105:923, 1993).

In this context, we have initiated the study of the promoter and regulatory sequences of mouse P-CD gene. A clone containing about 1 Kb of the 5' untranslated sequence of the P-CD gene has been isolated from a mouse genomic library. The analysis of the sequence of this region shows structural similarities with the upstream region of the mouse E-CD gene previously reported by Behrens et al. (P.N.A.S., 88: 11495, 1991): absence of a TATA box, presence of a CAAT box at -65 and a GC-rich region containing a potential SP1-binding element at -88 and a putative AP2-binding domain at -101; a second AP2 like sequence is located at +31 (Faraldo et al, J. Molec. Biol. 231, 935-941).

The functionality of the whole P-CD promoter and its different regulatory elements has been studied by transient transfection assays in expressing and non expressing cell lines using luciferase as a reporter gene. The results of this experiments suggest that the basal activity of the P-cadherin promoter in epidermal keratinocytes appears to be controlled by a combination of the proximal 5' AP2(-101), Sp1(-88) and CAAT(-65) elements. On the other hand the P-CD promoter is basically silent in spindle carcinoma cells (CarB).

Gel retardation and *in vitro* footprinting assays have been recently performed in order to try to identify the nuclear factors that can be interacting with the P-CD promoter in expressing and non expressing cells and therefore regulating its expression. Both types of experiments corroborated a clear interaction between SP1 factor and the postulated SP1-binding sequence. Concerning the CCAAT-box sequence, CP1 or a CP1-like protein could be involved in the expression of P-CD as deduced from the obtained results. However, no interaction was shown for the two predicted AP2-binding sequences when either techniques were used.

Similar experiments are presently starting to be developped in order to study the regulation of the E-CD expression in several cell lines representing different levels of malignancy in mouse skin carcinogenesis.

POSTERS

TITLE: p53 EXPRESSION IN EPIDERMODYSPLASIA VERRUCIFORMIS.

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Epidermodysplasia verruciformis (EV) is a rare autosomal recessive disease. Patients with EV have an abnormal predisposition to infection with specific types of human papilloma virus (HPV). EV patients also have a marked tendency for sun exposed lesions to degenerate into in situ and invasive squamous cell carcinoma (SCC). The p53 gene has been implicated in a wide range of human malignancies, including SCC of skin. Recent data have implicated the p53 protein in the response to DNA-damaging agents, such as UV ligth. We have investigated by immunohistochemistry the p53 expression in 12 benign and 14 malignant skin lesions from 5 EV patients. For comparison we also study 8 common warts, 8 flat wart, 6 bowenoid papulosis and 20 cases of Bowen´s disease, all of them from non-EV patients. We have used a mouse monoclonal antibody (DO7, Novocastra Laboratories Ltd.) which recognizes both the wild type and the mutant forms of the p53 protein.

Our results showed that most of the common warts, flat warts and cases of bowenoid papulosis from non-EV patients were negative for p53 immunostaining. In contrast, we observed positive p53 immunostaining in all the cases of flat warts, pityriasis-like warts and actinic keratosis in EV patients. The p53 immunoreactivity in malignant lesions was positive in 7/14 lesions from EV patients and in 11/20 lesions from non-EV patients.

Abnormal p53 protein expressed in benign lesions fron EV patients could be non functional or its suppressor function could be overcome by unknown mechanisms, leading to malignant transformation in some lesions located on sun exposed areas.

In order to find out the molecular basis of p53 overexpression, we are screening in benign EV lesions p53 exons 5 to 8 by a PCR-SSCP (polymerase chain reaction-simple strand conformation polymorphisme) approach as previously reported (Castresana et al., Int J Cancer 1993;55:562-5).

Decreased rate of malignant conversion of Sensitive Sencar Inbred (SSIN) papillomas using a three stage initiation/promotion/initiation carcinogenesis protocol. Gimenez-Conti, I.B., Winberg, L.D., Oubari, D. and Slaga, T.J., University of Texas M.D. Anderson Cancer Center, Smithville, Texas 78957

In the present study, we have further characterized the low rate of malignant conversion of the Sensitive Sencar Inbred (SSIN), compared to the parent Sencar mice. Groups of mice were initiated with a single topical application of either 2 or 10 nmole of 7,12-dimethylbenzanthracine (DMBA) followed by twice weekly applications of 0.5 µg 12-O-tetradecanoyl-13-acetate (TPA). After 20 weeks of promotion, mice were treated twice weekly with either 0.5 µg TPA or 20 mg benzoyl peroxide for 50 weeks; or 10 mmoles N-nitroso-N-ethylurea (ENU) for 4 weeks. The cummulative number of carcinomas observed in the SSIN strain was significantly lower compared to the parental Sencar mice regardless of the initiation dose of DMBA or the progression treatment (acetone, benzoyl peroxide, TPA, or ENU). It was also observed that the expression of markers of premalignant progression (GGT, keratin 13, and loss of keratin 1) was markedly suppressed in the SSIN. Further evaluation of the low papilloma-to-carcinoma conversion rates in SSIN mice relative to SENCAR mice was carried out using two complete carcinogenesis protocols. First DMBA, 50, 100, and 150 nmoles weekly for 8 weeks resulted in 7/30, 7/30, 10/30 cummulative carcinomas per group respectively at 52 weeks. A second complete carcinogenesis protocol consisted of ENU 0.5, 1.0, and 2.0 µmoles once weekly for 50 weeks. After 60 weeks, there are 1/30, 3/30, and 1/30 papillomas per mouse, respectively. No carcinomas were present. This study further confirms our earlier finding that SSIN appear to be highly sensitive to promotion but resistant to progression, suggesting that a gene(s) conferring susceptibility to tumor promotion was segregated from the gene(s) involved in tumor progression. Supported by NIH CA57596, CA43278, and Olga Keith Weiss Chair.

TGFß1 REGULATION OF CDC2 TRANSCRIPTION INVOLVES THE E1A ASSOCIATED PROTEIN p130

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Transforming growth factor ß 1 (TGFß1) is a potent inhibitor of epithelial cell proliferation. Keratinocytes expressing certain viral oncoproteins (E1A, LTAg, E7) have been found to be no longer responsive to the inhibitory effects of TGFß1. Since these viral oncoproteins are known to bind and thereby inactivate the product of the retinoblastoma susceptibility gene (p105Rb), p105Rb and related pocket proteins might be involved in TGFß1-induced growth inhibition. Rb-related proteins interact with the E2F family of cellular transcription factors, and this interaction can be disrupted by viral oncoproteins. Thus, E2F-regulated genes are likely cellular targets for the negative effects of TGFß1 on cell proliferation.

Expression of the human cdc2 gene can be induced by the viral oncoprotein E1A and suppressed by p105Rb, and both effects depend on the integrity of an E2F binding site present within the cdc2 promoter region. We have examined the effect of TGFß1 on cdc2 RNA levels in normal human foreskin keratinocytes and human keratinocyte cell lines immortalized by either the SV40 or HPV16 virus. TGFß1 suppressed both, cdc2 RNA levels and cdc2-associated H1 kinase activity in normal keratinocytes, while LTAg and E7 expressing keratinocytes were unresponsive. Kinetic gel shift assays carried out with whole cell extracts prepared from TGFß1 treated normal keratinocytes revealed a novel E2F-specific protein-DNA complex. Appearence of this complex coincided with cdc2 RNA down-regulation. Induction of this complex was not observed with the virally immortalized keratinocytes. As revealed by antibody competition experiments, this complex neither contains p105Rb nor p107. Reconstitution experiments using *in vitro* translated p130 showed that an E2F-p130 complex precisly comigrates with the TGFß1-induced E2F-complex.

The data are consistent with the model that TGFß1-induced inhibition of cdc4 kinase activity results in the accumulation of an inhibitory E2F-p130 complex at the cdc2 promoter, thus leading to cdc2 down-regulation in normal but not in virally immortalized keratinocytes. Transfection experiments are in progress to determine whether p130 directly down-regulates the cdc2 promoter.

A qualitative and quantitative protein database approach to the analysis of the protein phenotype of normal and SV40 transformed human keratinocytes

Eyðfinnur Olsen and Julio E. Celis.

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A qualitative and quantitative two-dimensional (2-D) gel database approach has been used to identify individual and groups of proteins that are differentially regulated in SV40 transformed human keratinocytes (K14). Five hundred and sixty [^{35}S]-methionine labeled proteins (462 isoelectric focusing, IEF; 98 nonequilibrium pH gradient electrophoresis, NEPHGE), out of the 3038 recorded in the master keratinocyte database, were cut from dry, silver stained gels of normal proliferating primary keratinocytes and K14 cells and the radioactivity determined by liquid scintillation counting. Two hundred and thirty five proteins were found to be either up (177) or downregulated (58) in the transformed cells by 50% or more, and of these, 115 corresponded to known proteins in the keratinocyte database (J. E. Celis et al., Electrophoresis 1993, 1091-1198). The lowest abundancy acidic protein quantitated was present in about 60,000 molecules per cell assuming a value of 10^8 molecules per cell for total actin. The results identified individual, and groups of functionally related proteins that are differentially regulated in K14 keratinocytes and that play a role in a variety of cellular activities that include general metabolism, the cytoskeleton, DNA replication and cell proliferation, transcription and translation, protein folding, assembly, repair and turnover, membrane traffic, signal transduction and differentiation. In addition, the results revealed several transformation sensitive proteins of unknown identity in the database as well as known proteins of yet undefined functions. Within the latter group, members of the S100 protein family - whose genes are clustered on human chromosome 1q21 - were among the highest downregulated proteins in K14 keratinocytes. Visual inspection of films exposed for different periods of time revealed only one new protein in the transformed K14 keratinocytes and this corresponded to keratin 18, a cytokeratin expressed mainly by simple epithelia. Beside providing with the first global overview of the functional changes associated with the transformed phenotype of human keratinocytes, the data strengthened previous evidence indicating that transformation results in the abnormal expression of normal genes rather than in the expression of new ones.

Title: E-cadherin and P-cadherin expression in basal cell carcinoma.

Authors: A. Pizarro, N. Benito, J. Espada, J. Palacios, A. Cano*, M. Quintanilla* and C. Gamallo.
Departamento de Anatomía Patológica, Hospital La Paz, Madrid; *Instituto de Investigaciones Biomédicas (CSIC-UAM), Madrid, Spain.

Cadherins (CDs) are calcium-dependent cell-cell adhesion molecules which play a crucial role in morphogenesis, as well as in the maintenance of adult tissue architecture. In human epidermis, E-CD is expressed in all the living layers, whereas P-CD expression is restricted to the basal layer.

We have studied E- and P-cadherin expression on a series of human basal cell carcinoma (BCC) of skin. This study included tumours of superficial, nodular and infiltrative histological types, which differ in both growth pattern and local invasiveness. Cadherin expression was studied using an immunohistochemical technique as previously reported (Pizarro et al., Br J Cancer 1994; 69: 157-62). We have employed mAbs specific for human E-CD (HECD-1) and human P-CD (NCC-CAD-299). Both antibodies were kindly provided by Prof. M. Takeichi.

We have found preserved E-CD expression in almost all cases of superficial and nodular BCC. E-CD immunostaining was reduced and heterogeneous in most cases of infiltrative BCC. In contrast, P-CD expression was preserved in most cases of BCC, including those tumours with an infiltrative growth pattern. Epidermis overlying BCC frequently showed strong P-CD immunostaining in the spinous layer. Thus, our results suggest different roles for E-CD and P-CD in human BCC of skin. Further investigations are needed to clarify the molecular basis and biological significance of increased P-CD expression in epidermis overlying BCCs.

TOWARDS THE SELECTION AND MOLECULAR ANALYSIS OF MUTATIONS OCCURRING IN VIVO IN MURINE KERATINOCYTES.

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The research aims are: i) to compare the incidence of tumor formation in mouse epidermis with the genetic load of mutations, ii) to compare mutagenesis and DNA damage repair in vivo and in vitro.
Initial experiments with a modified MCDB-153 showed that keratinocytes derived from newborn SKH hr/hr had a cloning efficiency of 0.5 - 1.5%. Keratinocytes derived from adult animals only had a reasonable cloning efficiency for the most basal cells. A cloning effciency of around 0.6% was obtained and the back of both a 19 weeks old animal and a 15 months old animal contained about 12000 clone forming cells which will be sufficient to assess mutant frequencies.
The size of the clones after 2 weeks cultivation was 5.7±1.1 cell generations. Thus about half of the clones measure 50 cells or more.
PCR of HPRT-cDNA of wild type cells indicated that amplified cDNA could be obtained starting with 300 cells. For PCR of half the cDNA 50 cells were sufficient to obtain a product.
The results obtained sofar indicate that it will be possible to determine the in vivo mutant frquency at the HPRT locus in the epidermis of the mouse and that it will be possible to analyse the mutation spectra if the growth of mutant cells during selection equals that of normal cells.

CONCLUSIONS

A. J. P. Klein-Szanto

The Workshop in Human and Experimental Skin Carcinogenesis was attended by scientists from round the world, including Spain, United Kingdom, USA, Holland, Denmark, France, Germany, Sweden and Switzerland. During three days, these researchers discussed in detail the mechanisms of skin cancer development in humans and experimental animals, emphasizing novel findings in molecular carcinogenesis.

In addition to six plenary sessions two discussion-sessions were held. During these last two sessions, several of the most debated and promising issues were openly discussed.

Most invited papers and posters presented data on the most widely used animal models, ie, chemical carcinogenesis in either conventional, transgenic or knock-out mice. Collectively, the information from these investigations indicate that very important genetic changes are paramount in the initiation, promotion and progression of mouse skin tumors. Among several genes that have been described as having a role in one or more of the stages of skin carcinogenesis are ras, fos, p53, NF-2 and cyclin D-1. In addition, several papers emphasized the importance of growth factors such as TGFα, and TGFβ1 as well as differentiation markers that are not always just passive biochemical indicators of skin tumor progression, eg. E. Cadherin, connexins, integrins, keratin 10, but could eventually through their loss or down regulation, play an important role in the acquisition of the malignant phenotype.

Many of the newer evidences on the function of oncogenes, tumor suppressor genes, as well as growth factors and differentiation markers were inferred from elegant experiments performed in recently designed transgenic and knock-out mice.

The relative value of rigid progression schemas was also emphasized by various speakers. The possibility that the cascade of molecular events during multistep carcinogenesis depends on the animal species and strain used and on the type of carcinogen and protocol of administration was also debated. For example, although Ha-ras mutations appearing during initiation and subsequent amplification during progression are important events in dimethyl-benzo(a)anthracene induced murine skin tumors, this is not seen in mouse skin neoplasia induced by other chemicals such as benzo(a)pyrene or by UV light. Other, still unknown, initiation events take place in the latter animal treatment protocols. In this context, it was emphasized that ras mutations are very seldom observed in human skin tumors. Conversely, p53 abnormalities seem to be quite frequent in all animal models described, and it is also the most important genetic event described in human non-melanoma skin cancer and its precursor lesions. Nevertheless, p53 is a relatively late event associated with conversion of papilloma to carcinoma and eventually to carcinoma progression in the mouse models, whereas in human skin it seems to be an early event seen in the preinvasive stage. The role of p53 abnormalities induced by UV light in human skin was described in several presentations, and its similarities and differences with p53 changes in animal models was discussed by numerous participants.

Malignant progression in human tumors is associated with chromosomal loss, e.g., chromosome 15; but different patterns have been detected in basal cell carcinomas (non-metastatic tumor) and in squamous cell carcinomas (metastatic tumors).

In addition to the numerous genetic abnormalities that play stage-specific roles in tumor development, some epigenetic events associated with epidermal proliferation were discussed, e.g., the putative function of phosphorylated PKC isoforms in skin tumor promotion.

It was clear from the presentation that the animal models, especially the new transgenic animals, knock-out mice and their crosses are and will continue to increase our knowledge on the mechanisms of carcinogenesis. It was also clear that these and other classical models of chemical carcinogenesis are a very good approximation to the human situation, but that more work is still necessary to produce relevant information that can clarify the mechanism of human and rodent skin carcinogenesis.

List of Invited Speakers

Workshop on

HUMAN AND EXPERIMENTAL SKIN CARCINOGENESIS

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HUMAN AND EXPERIMENTAL SKIN CARCINOGENESIS

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SERIE UNIVERSITARIA
by the
FUNDACIÓN JUAN MARCH
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Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on the Polymerase Chain Reaction.**
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264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

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Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 9th through the 11th of May, 1994, at the Instituto Juan March.

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