Environmental Health Perspectives
Vol. 93, 51–56, 1991

Differential Gene Expression during Multistage Carcinogenesis

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The use of the mouse skin multistage model of carcinogenesis has aided our understanding of critical target genes in chemical carcinogenesis. The mutagenic activation of the Harvey-*ras* proto-oncogene has been found to be an early event associated with the initiation of mouse skin tumors by the polycyclic aromatic hydrocarbon 7,12 dimethylbenz[*a*]anthracene and the pure initiator ethyl carbamate (urethane). In contrast to chemical initiation of mouse skin tumors, ionizing radiation-initiated malignant skin tumors have been shown to possess distinct non-*ras* transforming gene(s). Differential screening of cDNA libraries made from chemically initiated malignant skin tumors has been used to identify a number of cellular gene transcripts that are overexpressed during mouse skin tumor progression. These differentially expressed genes include β-actin, ubiquitin, a hyperproliferative keratin (K6), a gene whose product is a member of a fatty acid or lipid-binding protein family, and a gene called transin or stromelysin. The overexpression of the stromelysin gene, which encodes a metalloproteinase that degrades proteins in the basement membrane, is hypothesized to play a functional role in malignant tumor cell invasion and metastasis. We believe that the cloning, identification, and characterization of gene sequences that are differentially expressed during tumor progression could lead to the discovery of gene products that either play functional roles in skin tumor progression or in the maintenance of various progressive tumor phenotypes.

Introduction

During the process of either chemical or radiation carcinogenesis, the progression of target cells through a premalignant to malignant state is accompanied by a variety of morphological, cytological, and biochemical alterations. Presumably, these phenotypic changes result from either qualitative alterations in encoded gene products or changes in the levels of expression of cellular genes. One class of cellular genes that is known to be altered during carcinogenesis and is thought to play a functional role in tumor formation is the cellular proto-oncogenes (*1–3*). In addition to the proto-oncogenes, there is a class of cellular tumor-suppressor genes whose inactivation may be required for expression of the tumorigenic phenotype (*4*). Both chemical carcinogens and ionizing radiation are known to induce the types of mutations that have been observed to activate proto-oncogenes to transforming oncogenes and to inactivate tumor-suppressor genes. These mutations can lead to structural changes in encoded gene products or loss of normal control of expression of these genes.

The mouse skin model of multistage carcinogenesis is an ideal system in which to identify critical target genes for the action of chemical or physical carcinogens. The process of malignant tumor formation in the model can be subdivided into distinct stages operationally defined as initiation, promotion, and progression. A single subcarcinogenic dose of a chemical carcinogen or ionizing radiation can be delivered to the target tissue, epidermis, and no tumors will appear unless the initiator is followed by repeated doses of a tumor promoter such as a phorbol ester. The majority of the tumors arising from initiation-promotion protocols are benign papillomas. In the progression stage, the benign papillomas progress to form malignant squamous cell carcinomas (SCCs), and a number of agents including some initiators, certain peroxides, and ionizing radiation can enhance the progression of benign to malignant tumors (*5–7*). In addition, there are epidermal cell cultures that represent each of the stages in the formation of the malignant SCCs. Therefore, questions can be asked about this model concerning the timing of certain gene alterations including proto-oncogene activation, tumor-suppressor gene inactivation, and differential expression of other cellular genes. In addition, potential functional roles for altered gene expression in tumor progression can be assessed using mammalian expression vectors and the various epidermal cell cultures that represent the stages in tumor formation.

Over the last 6 years, a number of laboratories have used this multistage model to study proto-oncogene ac-

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tivation (*8–15*) and the differential expression (*16–21*) of tumor-associated genes. Here we summarize the findings that we have made concerning critical target gene alterations during multistage mouse skin carcinogenesis using both chemical initiation and ionizing radiation as an initiator.

Dominant Transforming Genes in Chemically and Radiation-Initiated Mouse Skin Tumors

Activated Harvey *ras* Oncogenes in Chemically Initiated Mouse Skin Tumors

In collaboration with Balmain's laboratory (*9*), we have demonstrated the presence of dominant transforming activity in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated,12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted papillomas, and malignant SCCs using the NIH 3T3 transfection focus assay. This dominant transforming activity was attributed to the activation of the Harvey *ras* (Ha-*ras*) gene, and the activation was considered an early event associated with DMBA initiation because the activated oncogene was shown in benign papillomas. Subsequently, Balmain's laboratory (*10*) demonstrated that more than 90% of the DMBA-initiated tumors had a specific A:T transversion mutation at the second nucleotide of codon 61 of the Ha-*ras* gene. This was demonstrated using DNA sequencing, restriction-fragment-length polymorphisms (RFLP), and oligonucleotide probing. The A:T transversion mutation resulted in the creation of a new *Xba*I site, which was detected by Southern blot analysis. It was shown using RFLP analysis that the mutation was heterozygous in most papillomas and homozygous and amplified in some but not all of the SCCs.

More recently, work in our laboratory at the University of Arizona Medical School has focused on dominant transforming activity in skin tumors initiated by the carcinogen ethyl carbamate (urethane) (*12*). Urethane is a pure initiator of mouse skin tumors that requires metabolic activation to yield an ultimate carcinogenic form, most likely an epoxide (*22–24*). Using the NIH 3T3 focus-forming assay, dominant transforming activity was detected in DNA isolated from SCCs initiated with urethane. Rearranged and amplified copies of the c-Ha-*ras* gene were detected in DNA-isolated transformant cell lines, indicating that an activated Ha-*ras* gene had been transferred to the NIH 3T3 recipient cells. Analysis of p21ras from the transformant cell lines suggested that the activating *ras* mutation was present in codon 61. Ultimately, the Ha-*ras* gene was shown to be activated by a specific A:T transversion mutation at the second position of codon 61. This mutation was detected in both benign papillomas and SCCs, suggesting the activation occurred early in tumor development.

Recent results (*25*) concerning urethane-induced lung tumors in A/J mice showed evidence for consistent activation of the Kirsten *ras* gene by the same A:T transversion mutation at codon 61. In addition, vinyl carbamate, a proximate carcinogen that is a probable intermediate metabolite of urethane, produced mouse hepatomas (*26*) with the same codon 61 A:T transversion in the c-Ha-*ras* gene. Therefore, three different mouse tumor types induced with urethane or its probable intermediate metabolite contained a high frequency (though not exclusively) of *ras* gene activation by the same A:T transversion mutation at codon 61. The model of direct interaction of tumor initiators with specific target bases in the *ras* proto-oncogene cannot be directly related to what is known about major DNA adducts formed with urethane or vinyl carbamate. Following administration of urethane or vinyl carbamate, 7-(2-oxoethyl)guanine was the only major adduct identified in rat liver (*24,27*). It has been proposed that this adduct may isomerize to yield an O^6,7-(1′hydroxyetheno)guanine adduct that potentially could lead to a G:A transition mutation. Clearly, further work needs to be done in this area to determine why the observed A:T transversion mutation is predominant despite the lack of evidence for DNA-deoxyadenosine adducts.

Distinct Non-*ras* Transforming Genes in Ionizing Radiation-Initiated Mouse Skin Tumors

In addition to studying chemical carcinogen-initiated mouse skin tumors, we have focused on ionizing radiation-initiated, TPA-promoted skin tumors and dominant transforming genes. We found that ionizing radiation can act as a weak initiator (*28,29*) of malignant skin tumors (i.e., SCCs) when initiation is followed by TPA promotion. In addition, we observed that ionizing radiation was capable of inducing basal cell carcinomas, a tumor histology not seen with chemical agents in the mouse skin system. We have also observed that fractionated doses of accelerated electrons were effective in the third stage of tumor progression. DNAs from mouse skin tumors initiated with ionizing radiation including papillomas, SCCs, basal cell carcinomas (BCCs), and pilomatrixomas demonstrated dominant transforming activity by the production of transformed foci in the mouse recipient line, NIH 3T3 (*30*). Dominant transforming activity was not found in DNA from normal epidermis or from the corresponding liver.

The NIH 3T3 transformants induced with SCC DNA grew in soft agar and formed tumors in nude mice. Southern blot analyses of primary NIH 3T3 transformant DNAs carrying transforming genes from radiation-initiated SCCs indicated that the oncogenes responsible for the transformation of the recipient cells were not Ha-*ras*, Ki-*ras*, or N-*ras*, nor were they *erb*B, B-*lym*, *met*, *neu*, or *raf*. The transforming gene(s) transferred by DNA from four SCCs were further characterized by determining their sensitivity to digestion with a series of restriction enzymes. The results of these

experiments indicated that there were at least three different transforming genes present in four SCCs initiated with ionizing radiation.

Our data suggest that the target gene(s) for oncogenic activation are different for chemical carcinogens and ionizing radiation. Support for this finding has recently been presented by Borek et al. (*31*) as well as by Krolewski and Little (*32*). These workers have also detected distinct non-*ras* transforming genes in ionizing radiation-transformed rodent cells in culture. Perhaps it is not surprising that activation of the *ras* proto-oncogenes, and in particular the Harvey *ras* oncogene, was not identified with radiation-initiated mouse skin tumors as was consistently observed with chemically initiated mouse skin tumors (*8–12*). The *ras* family of oncogenes is activated by point mutations, and the chemical initiating agents or their active metabolites are known to be relatively efficient point mutagens.

In contrast, ionizing radiation is a relatively weak point mutagen and instead induces larger genomic alterations. Goodhead (*33*) has compared the cross sections for ionizing radiation-induced mutations and *in vitro* transformation. From these studies he has suggested that the target for transformation is larger than a single gene and smaller than a chromosome and may involve more than one gene or chromosome. Rauth (*34*) has suggested that direct activation of a transforming gene may not be occurring. He speculated that a variety of genes that control stability of DNA or fidelity of DNA replication may be damaged by radiation and increase the probability of errors in DNA during subsequent rounds of DNA replication. Therefore, the likelihood of a second step leading to activation of a transforming gene or oncogene would increase. Our finding of at least three different transforming genes in four radiation-initiated malignant skin tumors supports Rauth's hypothesis in that multiple transforming genes are likely to result from secondary DNA damage due to the direct effects of ionizing radiation on some cellular genes that regulate DNA replication and repair or genomic stability.

Differential Gene Expression during Mouse Skin Carcinogenesis

Expression Pattern of the *mal* Genes in Benign and Malignant Skin Tumors

In addition to studying dominant transforming genes as target genes activated during mouse skin carcinogenesis, we have also identified and characterized cellular genes whose expression is altered during tumor development (*15*). To achieve this goal, we have used differential screening of cDNA libraries that were made from polyA+ RNA isolated from SCCs induced by DMBA initiation and TPA promotion. The libraries were screened using cDNA probes made from RNA isolated from normal epidermis and SCCs. We isolated six cDNAs (*mal*1 to *mal*6) that identified distinct RNA transcripts that were overexpressed at different stages during skin tumor progression. To investigate a potential role of the *mal* genes in the process of skin carcinogenesis, we asked whether there was a correlation between the stage of tumor development and the level of expression of the different *mal* genes. In addition, we sequenced the cDNAs for the *mal* genes and searched gene sequence data bases for homology or identity with known genes.

Our work to date has focused on the *mal*1 to *mal*4 genes. The *mal*1 gene was found to be overexpressed in both benign and malignant skin tumors in comparison to normal adult epidermis (*15,35*). Sequencing of the *mal*1 cDNA has revealed extensive sequence homology to a family of low molecular weight, hydrophobic, ligand-binding proteins. This family includes a differentiation-associated protein in adipocytes (adipocytes lipid-binding protein), mouse myelin P2 protein, a polypeptide growth inhibitor purified from bovine mammary gland, fatty-acid-binding protein and cellular retinol-binding protein (CRBP) [reviewed in Demmer et al. (*36*)]. These low molecular weight, cytosolic, nonenzymatic proteins form a multigene family of proteins presumably derived from a common ancestral gene. These proteins bind fatty acids or retinoids and are presumed to function in some aspect of intracellular lipid metabolism. Some of them may play a functional role in proliferation and differentiation.

Recent studies suggest a role for cellular retinol-binding protein in carcinogenesis (*37–39*). Several workers have shown that squamous cell carcinomas of the head and neck contained increased levels of cellular retinol-binding protein compared to normal tissue. Since retinol inhibits terminal differentiation of keratinocytes, it has been suggested that the increased CRBP levels in squamous cell carcinomas may cause the decreased terminal differentiation observed in these carcinoma cells as compared with normal keratinocytes. Whether the tumor-specific overexpression of the *mal*1 gene may be linked to similar defective processes involved in tumor de-differentiation and cellular proliferation has to be determined by further experiments.

We have found that the *mal*2 gene transcripts are also overexpressed in both benign and malignant skin tumors. Our expression data have shown that there is a higher steady-state level of *mal*2 transcripts in benign papillomas that are autonomous (i.e., no longer require promoter treatment) compared to papillomas that are dependent on continued tumor promoter treatment (*35*). Sequencing of a *mal*2 cDNA and a *mal*2 genomic clone has recently revealed identity with the sequence for a mouse hyperproliferative keratin, K-6 (D. R. Roop, personal communication). The *mal*3 cDNA used in probing of Northern blots has revealed multiple hybridizing bands. The intensity and pattern of these *mal*3-related bands or transcripts changed during tumor development. In normal adult epidermis, low steady-state lev-

els of three transcripts were detected (i.e., 1.3, 2.3, and 2.9 kb). In benign papillomas, the 1.3 and 2.3 kb transcripts were overexpressed, whereas the largest 2.9 kb transcript was not detectable. In malignant SCCs, this largest transcript related to *mal3* was always overexpressed, whereas the 2.3 kb transcript either was absent or was expressed at a lower level.

Sequencing of a full-length *mal3* cDNA has shown identity with the highly conserved coding region of the ubiquitin gene (*40*). Our cDNA sequence contained 96 bases of 5′ noncoding region and 152 bases of 3′ noncoding region. We have used both 5′ and 3′ specific oligonucleotide probes to determine that the cDNA that we cloned and sequenced corresponds to the ubiquitin gene (which encodes four tandem open reading frames of the ubiquitin monomeric protein) whose transcribed message is the 1.3 kb transcript that is overexpressed in both papillomas and SCCs compared to normal epidermis (*21*). One of the six *mal* cDNA sequences, *mal4*, detected 1.9 kb transcripts that were expressed at levels 10-fold higher in SCCs in comparison to normal epidermis. A full-length cDNA for *mal4* was obtained from a λgt10 cDNA library made from an SCC-producing cell line, PDVc57. DNA sequencing of this full-length *mal4* cDNA revealed identity with the mouse β-actin cDNA. Southern analysis of DNAs from normal epidermis, papillomas, and SCCs showed no evidence for amplification or gross rearrangement of the β-actin gene during tumor progression.

The RNA-RNA hybrid protection assay was used to screen for the expression of mutated β-actin(s) in mouse skin tumors. No evidence for a mutation was obtained in the benign and malignant skin tumors that were examined. Fluorescence microscopy of tumor sections stained with rhodamine-conjugated phalloidin showed a peripheral pattern of F-actin localization with no gross differences between papillomas and SCCs. We also found approximately equal amounts of β-actin protein detected by two-dimensional gel electrophoresis and extracted from normal epidermis, papillomas, or SCCs. These results indicated that the overexpression of β-actin RNA in SCCs did not result in an increased steady-state level of β-actin protein. This would indicate that translational or posttranslational mechanisms may be functioning to maintain a relatively constant cellular concentration of actin in the presence of high levels of β-actin-specific RNA. It is also possible that in SCCs and some papillomas there is an increased synthesis of β-actin protein (paralleling the increased mRNA) but that there is increased degradation of β-actin protein such that the steady-state level of protein is not altered. An increased turnover of actin has been reported in cultured fibroblasts from individuals susceptible to dominantly inherited cancer (*41*). Because these predisposed fibroblasts show reduced and disorganized microfilaments, features that have been associated with increased motility of tumor cells in culture and increased metastatic potential *in vivo*, it is clear that alterations in the properties or metabolism of actins can have profound effects on cellular phenotypes.

Expression Pattern of the Transin Gene during Mouse Skin Tumor Progression

The rodent transin gene encodes an oncogene-inducible protein that has been shown to be a rodent homologue (*16*) of an extracellular matrix-degrading metalloproteinase known as stromelysin (*42*). The rat transin or stromelysin cDNA was originally cloned because of its selective expression in polyoma virus-transformed rat fibroblast cells and lack of expression in untransformed parental cells (*43*). Later studies indicated that stromelysin was present in cell lines transformed by a number of different oncogenes. These results prompted us to study the expression pattern of the stromelysin gene in mouse skin tumors produced in an initiation-promotion protocol (*16*).

When RNA was isolated from tumors resulting from DMBA initiation and TPA promotion, stromelysin transcripts were detectable in 73% of the SCCs, but only 6% of the papillomas expressed low levels of stromelysin. Similar results were obtained with tumors initiated by *N*-methyl-*N*-nitroso-*N*′-nitroguanidine (MNNG) and promoted with TPA. When mice were treated with repeated applications of MNNG, a protocol which produces malignant tumors with a high probability of invading and metastasizing, 100% of the malignant tumors contained very high levels of stromelysin transcripts (*44*). In relation to the overexpression of the stromelysin gene in invasive and metastatic skin tumors, it is of interest that stromelysin when activated can degrade fibronectin, laminin, proteoglycans, gelatins, and to some extent collagen types III, IV, and V (*45*). These protein substrates are found in basement membrane that separates the epidermis from the dermis and must be transversed by malignant skin tumor cells for both invasion and metastasis to occur.

In addition to finding constitutive expression of the stromelysin gene in malignant skin tumors, we have also observed transient induced expression of the gene in phorbol-ester-treated normal mouse epidermis (*17*). This response in terms of steady-state level of stromelysin message is transient, with peak levels occurring 12 to 18 hr after TPA treatment and reduced to background levels by 24 hr. Stromelysin expression is also stimulated by the incomplete second-stage tumor promoter 12-*O*-retinoylphorbol-13-acetate (RPA) but not induced by ethylphenyl propiolate, a nontumor promoting, hyperplastic agent. The stromelysin transcripts are localized to the basal cells of the TPA-stimulated epidermis. These results suggested that the transient induction of stromelysin during tumor promotion is a phorbol-ester-specific response and that it is not a result of the proliferative response to tumor promoters.

Our concept of the role of stromelysin in tumor progression is summarized as follows. Initiation of mouse skin with chemical carcinogens results in the activation of the Harvey *ras* oncogene in a small population of epidermal cells. These cells with the activated *ras* oncogene are thought to be "initiated" cells that undergo clonal expansion under the influence of a tumor pro-

moter to give rise to a benign papilloma. Repeated treatment with the tumor promoter also results in repeated, transient elevations in stromelysin. Papillomas can be classified by their behavior after withdraw from tumor promoter treatment. "Dependent" papillomas are dependent on continuous promoter treatment or they will regress, whereas "autonomous" papillomas are no longer dependent on promoter treatment. Since a small percentage of papillomas contain stromelysin transcripts, we speculate that these tumors may have been "autonomous" and premalignant. Stromelysin expression may be constitutive in these premalignant tumors and no longer dependent on tumor promoter treatment. Conversion to malignant tumors is usually accompanied by constitutive expression of stromelysin. The reason for the constitutive expression of stromelysin in malignant SCCs is not because of either amplification or gross rearrangement of the stromelysin gene (*44*). Since the tumors with the greatest probability of becoming invasive and metastatic have the greatest probability of expressing high levels of stromelysin, we speculate that this enzyme plays a causal role in promoting invasion through basement membrane barriers. Experiments being conducted in our laboratory are testing the hypothesis that the overexpression of the stromelysin gene is both necessary and sufficient for progression of benign papilloma cells to malignant tumor cells and the hypothesis that the overexpression of this gene is necessary but not sufficient for progression.

The authors are indebted to several colleagues who carried out part of the studies described in this paper, in particular, Lynn Matrisian, Larry Ostrowski, Debbie Jaffe, Keith Bonham, Joanne Finch, and Karen Holladay. The authors also acknowledge Margaret Deering for expert secretarial assistance. This work was supported in part by grants CA-42239 and CA-40584 from the National Institutes of Health.

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