Oncogene Activation and Tumor Suppressor Gene Inactivation during Multistage Mouse Skin Carcinogenesis

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Abstract

The mouse skin multistage model of carcinogenesis is an ideal system in which to study questions related to the timing of oncogene activation and inactivation of tumor suppressor genes. A number of laboratories have shown that an early event associated with chemical initiation of mouse skin tumors involves activation of the Harvey-ras oncogene. To approach the question of timing of loss of tumor suppressor genes in skin carcinogenesis, we have utilized a model system developed by Kulesz-Martin in which cloned mouse keratinocytes were initiated with DMBA and variant clones with benign or malignant phenotypes were developed. We have generated somatic cell hybrids between the parental clone and the variants to study the potential loss of tumor suppressor activity during the progression of cells from the initiated to benign and to the malignant phenotypes. Somatic cell hybrids generated between the parental, normal cell strain (i.e., 291) and a malignant cell variant (i.e., 09), which produces moderately differentiated squamous cell carcinomas (SCCs), failed to produce tumors indicating tumor suppressor activity in the 291 cells. The 291 cell and a benign papilloma producing variant (i.e., 09) were able to partially suppress in hybrids the tumorigenicity of another malignant cell line (i.e., 03) which produces poorly-differentiated SCCs. Suppression of tumor suppressor activity by the benign tumor cell, 291, was less than that seen with the normal cell, 291. These results indicated two potentially different suppressor activities were inactivated during progression of normal 291 to malignant 09 cells. We have also obtained evidence that constitutive AP-1 activity plays a role in the maintenance of the malignant phenotype of SCC cell lines. Two different SCC cell lines, 308 10GyS and PDV, demonstrated constitutive AP-1 activity. To examine the role of this activity in malignant progression, we stably expressed a transactivation deletion mutant of the human c-jun gene in these cell lines. Expression of this mutant c-jun protein blocked transcriptional transactivation of AP-1 responsive reporter CAT constructs driven by jun, human collagenase, and the mouse stromelysin promoters. These malignant cells were not only inhibited in their AP-1 transactivation response, but also in their ability to form SCCs upon s.c. injection into athymic nude mice. These results support the idea that inhibition of AP-1-mediated transcriptional transactivation is in some cases sufficient to suppress the tumorigenic phenotype of malignant mouse epidermal cells.

Introduction

The development of fully malignant tumor cells from target stem cells involves for most epithelial systems stable intermediate stages. The development of each stage is accompanied by a variety of biochemical, morphological, and cytological changes. These phenotypic alterations result in turn from either qualitative or quantitative changes in certain cellular genes. These known cellular genes include oncogenes (1–3), tumor suppressor genes (4), and effector genes (5) whose expression is regulated by oncogenes or tumor suppressor genes. The oncogenes are derived by mutational activation of cellular protooncogenes and the tumor suppressor genes are inactivated also by mutation. Chemical carcinogens as well as radiation are known to induce the types of mutations found to activate protooncogenes and inactivate tumor suppressor genes. Structural changes and loss of normal regulation of expression of these genes could result from these carcinogen-induced mutations. An important question is whether the chemical carcinogens directly induce the mutations found in critical genes or whether the carcinogens are indirectly involved in selection for cells containing spontaneously induced target gene mutations.

The mouse skin model of multistage carcinogenesis is a system with numerous advantages for studying the timing of oncogene activation and tumor suppressor gene inactivation. The development of malignant squamous cell carcinomas in this model involves three distinct operational stages including initiation, promotion, and progression (6, 7). Initiation and promotion lead to the development of benign papillomas some of which are premalignant and will progress to malignancy. The third stage of malignant progression involves the induced conversion of papillomas to squamous cell carcinomas (8–10). An advantage to this carcinogenesis model are mouse keratinocyte cell cultures that represent each of the intermediate stages in tumor development. Using both the animal and cell culture models of mouse skin carcinogenesis, researchers have begun to delineate critical gene alterations that occur at each of the stages of mouse skin tumor development. A very early event associated with chemical initiation is the activation of the Harvey-ras oncogene by specific point mutations (11–13). Amplification of the mutant Harvey-ras allele and loss of the normal Harvey-ras allele occur at late stages in malignant progression (14). Inactivation of the p53 tumor suppressor gene is another late event in tumor progression (15–17). At the level of chromosome alterations trisomy of chromosome 6 and 7 are events that occur in the development of progressed or late papillomas and they also occur in SCCs (18). Loss of heterozygosity has been demonstrated at loci on the distal arm of chromosome 7 in SCCs (19). This could be indicative of inactivation of tumor suppressor genes located in this region. Finally, we and others (20–24) have found enhanced expression of certain cellular genes at specific stages in the development of mouse skin tumors and more recently enhanced activity of certain transcription factors which may regulate the expression of these cellular genes.

Other paradigms of carcinogenesis such as human colorectal cancers have indicated that multiple tumor suppressor genes are inactivated at specific stages in tumor development. In the mouse skin model there is little information concerning the number and timing of inactivation of tumor suppressor genes. We have utilized a mouse keratinocyte cell culture model of carcinogenesis and somatic cell hybrids to address these questions. A related question concerning the functional role of constitutive AP-1 transactivation in the maintenance of the malignant phenotype is also addressed.

Results

Induced Differentiation and Tumor Suppressor Gene Inactivation in Mouse Keratinocyte Somatic Cell Hybrids. While it has been clearly shown that many animal and human malignancies have lost tumor suppressor activities and genes, the exact timing of inactivation during the multistep process of malignant tumor development has not been defined. We have used a mouse keratinocyte single cell lineage
model to identify temporal and functional importance of tumor suppressor activity loss in multistage carcinogenesis. The mouse keratinocyte single cell lineage model of carcinogenesis was developed by Kulesz-Martin (25–28). A nontumorigenic epidermal cell strain (i.e., 291) that was sensitive to calcium-induced terminal differentiation was treated with 7,12-dimethylbenz[a]anthracene. Three phenotypic variants that were resistant to calcium-induced terminal differentiation were isolated and they included: (a) a benign papilloma forming cell line 291.09RAT (09); (b) a highly undifferentiated, locally invasive carcinoma cell line 291.03RAT (03); and (c) a less malignant moderately differentiated carcinoma cell line 291.05RAT (05).

The parental 291 cells and the three variant cell lines were stably transfected with various antibiotic resistance genes to allow selection of somatic cell hybrids. By creating somatic cell hybrids between the nontumorigenic 291 cells and each of variants, we were able to assess the timing of tumor suppressor activity loss in this multistage model of carcinogenesis. We have generated the following somatic cell hybrids: nontumorigenic (291) X benign (09); nontumorigenic (291) X malignant (03); nontumorigenic (291) X malignant (05); benign (09) X malignant (03); and malignant (03) X malignant (05). Unhybridized cell lines and the somatic cell hybrids were injected s.c. into athymic nude mice to determine the malignant phenotype of these cells. The malignant but not the benign tumor cells form tumors upon s.c. injection (26). All of the somatic cell hybrids showed varying degrees of suppression of tumor formation.

The nontumorigenic cells completely suppressed tumor formation of the well differentiated malignant 05 cells. In contrast, the nontumorigenic cells partially suppressed the tumorigenicity of the anaplastic malignant cells, 03. The benign tumor cells also partially suppressed the tumorigenicity of the malignant 03 cells; however, the degree of suppression was less than that seen with the nontumorigenic 291 cells. Interestingly, the less malignant, moderately differentiated malignant cell line suppressed the tumorigenicity of the anaplastic malignant 03 cell line. In comparison to the parental malignant cell lines, all of the hybrids demonstrated fewer local metastases and, when tumor formation was not suppressed, the resulting tumors tended to be more highly differentiated. In addition, injection sites in which tumors failed to form were devoid of injected hybrid cells detectable by polymerase chain reaction amplification of selectable marker genes. We have obtained gene expression data supporting the idea that suppression of tumorigenicity in the hybrids involved induced terminal differentiation. The nontumorigenic 291 cells but not the malignant 03 cells expressed the terminal differentiation-specific keratin gene, K1 (29). We found that the 291 x 03 hybrid cells in culture reexpressed the K1 keratin gene.

**Constitutive AP-1 Activity Plays a Functional Role in Maintenance of the Malignant Phenotype of Squamous Cell Carcinoma Cells.** Aberrant transcriptional regulation is now clearly associated with deregulated gene expression and carcinogenesis. One of the transcription factor complexes for which there is a role in carcinogenesis is the AP-1 complex consisting of homodimers of jun family members or heterodimers between jun and fos family members (30–33). AP-1 is known to be activated to bind and transactivate specific consensus elements in certain cellular genes. In the mouse skin model of carcinogenesis repeated applications of the tumor promoting agent TPA to initiated skin results in the development of benign papillomas and transiently induces the expression of genes in the epidermis known to have the AP-1 consensus cis-element (34). In addition, TPA treatment of cultured epidermal cells leads to a rapid and transient increase in AP-1 DNA binding activity as well as AP-1 transactivating activity. A role for constitutive AP-1 activity was initially established for induction of the malignant phenotype in the epidermal system when Greenhalgh and Youspa (35) showed that expression of the deregulated viral-fos gene in benign papilloma cells already expressing an activated Harvey-ras oncogene caused these cells to progress to malignancy (35). We have also shown that malignant SCC cells constitutively express cellular genes that are known to be regulated by TPA and are known to contain AP-1 cis regulatory elements. Finally, we have shown that an ionizing radiation-induced malignant variant cell line (i.e., 308 10Gy5) of a benign papilloma cell line (i.e., 308) (36) expresses constitutive AP-1 DNA binding and transactivating ability. In addition, another malignant SCC producing cell line, PDV, constitutively expresses AP-1 activity. Taken together, these results indicate that acquisition of constitutive AP-1 activity may be of significant mechanistic importance in the promotion and progression of skin cancer. We have hypothesized that inhibition of AP-1 mediated cellular events might result in the blocking of the malignant phenotype. To experimentally approach this hypothesis we have expressed in malignant mouse epidermal cell lines a c-jun deletion mutant protein, TAM-67, that has been shown to act as a dominant negative transcription factor (37, 38).

The TAM-67 encoded mutant protein is lacking its transcriptional activation domain; however, it retains the leucine zipper DNA binding domains. It has been shown to complex with both c-jun and c-fos proteins (38); however, these complexes do not transactivate an AP-1 consensus element. In addition, the mutant c-jun protein has been shown to act in a dominant negative manner to inhibit c-jun/ras and c-fos/ras mediated cotransformation of rat embryo cells (37). In our studies the TAM-67 protein was stably expressed using a mammalian expression vector in two malignant keratinocyte cell lines, 308 10Gy5 and PDV. We found that expression of the TAM-67 mutant protein blocked transcriptional transactivation of AP-1 responsive reporter CAT constructs driven by the jun promoter, the human collagenase promoter and the mouse stromelysin promoter. Constitutive and inducible transactivation from these promoters was inhibited in TAM67 transfected cells but not in vector only transfected cells or malignant parental cells. Malignant mouse epidermal cells which stably expressed the mutant c-jun protein were not only inhibited in their AP-1 transactivation response but also in their ability to form s.c. tumors when injected into athymic nude mice. Our experimental results indicate that inhibition of AP-1-mediated transcriptional transactivation alone is sufficient to suppress the tumorigenic phenotype of malignant mouse keratinocyte cell lines. These results support the idea that constitutive AP-1 activity could play a functional role in the conversion to and maintenance of the malignant phenotype of SCC cells.

**Discussion**

Studies of the somatic cell hybrid have revealed the presence of multiple tumor suppressor genes in a single lineage model of multistage carcinogenesis. These studies have also helped to place a functional and temporal significance to loss of tumor suppressor activities in the development of epithelial tumors. We have obtained evidence in our keratinocyte model that a potential mechanism for suppression of tumorigenicity is the induction of terminal differentiation. In the majority of the tumor/normal cell hybrids tested, increased extracellular calcium concentrations induced a differentiated phenotype in the cultured cells similar to the response of normal cells. We detected the expression of the terminal differentiation specific keratin gene, K1. Expression of this gene indicated that a population of hybrids had regained the ability to terminally differentiate. In a similar study, Peahl and Stanbridge (39) have demonstrated that when HeLa cervical carcinoma cells were fused to normal human keratinocytes, the loss of tumorigenic potential was associated with the induction of differentiation in vivo. In addition, researchers have obtained evidence from tumor, normal fibroblast hybrids that the tumor suppression is con-
comminant with fibroblast differentiation (40). In these particular examples of somatic cell hybrids including our own hybrids, the differentiation program of the nontumorigenic cell plays a dominant functional role in inducing terminal differentiation.

Our data suggest that there is inactivation of two suppressor activities in the development of the malignant, anaplastic 03 tumor cells. One activity appears to be lost in the progression of nontumorigenic 291 cells to the benign tumor cells, 09, and a second activity may be lost in the progression of benign 09 to malignant 03 cells. Our data support the idea that tumor suppressor gene loss is a contributory genetic event in the formation of benign keratinocyte tumors. Our results also indicate that inactivation of tumor suppressor activity is a relatively early event in the formation of benign keratinocyte tumors.

Our studies related to critical molecular events in the progression of benign to malignant keratinocyte tumors have indicated a functional role for constitutive AP-1 transactivation activity in the establishment or maintenance of the malignant phenotype. We have used a dominant negative mutant c-jun protein (i.e., TAM 67) to block AP-1 activity in malignant SCC cell lines. Among the TAM67 transfected SCC cells we observed specific inhibition of both constitutive and inducible AP-1 mediated transcriptional activation, a decrease in the steady state levels of transcripts for certain AP-1 responsive, effector genes, and have demonstrated significant suppression of tumorigenicity. The expression of dominant negative transcription factors to reverse a malignant phenotype has been shown by other investigators (41–43).

Both c-fos and c-jun mutant proteins have been shown to act as inhibitors of the transformed phenotype. The TAM 67 mutant c-jun protein utilized in our studies have previously been reported to inhibit c-jun/c-fos mediated transactivation of rat embryo cells (37). In our in vitro studies, however, are the first to demonstrate that a c-jun mutant protein missing the transactivation domain when expressed in malignant epithelial cell lines inhibits their ability to form tumors in an athymic mouse. The anticipated mechanism of action of this transactivation dominant mutant c-jun protein is to block or quench endogenous cJun/AP-1 from binding and transactivating gene promoters containing TREs (38). Since the TAM67 protein still retains the DNA binding and leucine zipper domains it is possible for this protein to dimerize, occupy the TRE sites on critical genes, block access by normal jun or jun/fos dimers, and not transactivate. It is also possible that TAM 67 could dimerize with jun and/or fos, occupy the TRE sites but not transactivate. This would be a quenching mechanism.

Finally, there is the possibility that TAM 67 could mediate its inhibitory effects through non-DNA binding mediated squelching mechanisms. We are presently investigating these alternative mechanisms in our model system.

In conclusion, we have obtained evidence in cell culture models of malignant transformation of mouse keratinocytes for multiple genetic alterations that occur at different stages in the development of malignant tumors. A unique finding has been evidence for inactivation of tumor suppressor activity in the development of benign tumors and inactivation of a second activity in the progression of benign to malignant tumors. In a different model system we have obtained evidence that constitutive AP-1 transactivation activity plays a functional role in at least maintenance of the malignant phenotype. Whether inactivation of the second tumor suppressor activity is involved in the constitutive activation of AP-1 is not known; however, this question is the subject of further investigations.

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