Molecular Events Involved in Ionizing Radiation Induced Skin Carcinogenesis

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The process of mouse skin tumor formation is subdivided into three operational stages. These stages include initiation, promotion and progression. Ionizing radiation has been found to be a weak initiating agent in the production of malignant squamous cell carcinomas, a complete carcinogen and an agent effective in causing tumor progression. Four skin tumor histologies have been seen with ionizing radiation: benign papillomas, squamous (SCC) and basal (BCC) cell carcinomas and fibrosarcomas. Distinct non-ras transforming genes have been detected in radiation initiated SCCs. A benign papilloma cell line (308) was used as a model system to study ionizing radiation induced progression. A variant 308 cell line (308 10 Gy 5) derived by irradiation of the parental 308 cell has been characterized. The 308 10 Gy 5 cells unlike the parental 308 cells form malignant tumors in athymic nude mice upon subcutaneous injection. The variant 308 10 Gy 5 cells unlike the parental cells also show by northern analysis high steady state levels of the following gene transcripts: stromelysin, metallothionein II A and the proto-oncogenes c-fos and c-jun. Transient transfection studies with a chimeric mouse stromelysin promoter sequence upstream of a chloramphenicol (CAT) reporter gene into 308 and 308 10 Gy 5 cells indicated that the stromelysin promoter was constitutively active in the 308 10 Gy 5 but not in the 308 cells. The ability to divide the process of carcinogenesis into multiple stages in the mouse skin mode has facilitated mechanistic studies that may elucidate the molecular pathways involved in radiation induced tumor development.

INTRODUCTION

Most cancers develop as a result of a succession of steps occurring over a long latency period. During tumor progression in many human and animal models an important feature is the appearance of premalignant benign lesions, a small proportion of which progress to malignancy (1). In vitro and in vivo multistage models of carcinogenesis have been developed in a number of tissues and species (2–5). This multistage process can be subdivided operationally into three stages: initiation, promotion and progression. In the mouse skin model, tumors are induced by the sequential application of a single subcarcinogenic dose of a complete carcinogen followed by repeated treatments with a cocarcinogenic, hyperplastic tumor promoter. In a two stage protocol the majority of the tumors are benign papillomas.
and a small percentage of the benign tumors progress to malignant SCCs. Hennings et al. (6) have demonstrated that the tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) is not very effective in the third stage of tumor progression. These investigators have shown that various initiators including N-methyl-N-nitroso-N'-nitro-guanidine (MNNG), urethane and 4-nitroquinoline oxide (4NQO), are effective in the third stage suggesting that malignant conversion requires an additional step(s), possibly a mutation or a clastogenic event. O'Connell et al. (7) have recently shown that certain peroxides such as hydrogen peroxide and benzoyl peroxide can also enhance the conversion of benign papillomas to SCCs. Both of these peroxides are strong clastogens. Humans are exposed to a variety of clastogenic factors and mutagens many of which may be important in the multistage process of tumor formation. The clastogenic and mutagenic effects of ionizing radiation along with its wide use in the medical field have prompted our studies to determine if ionizing radiation can initiate either benign or malignant skin tumors in the mouse (8) and whether ionizing radiation is effective in the third stage of progression of benign papillomas to malignant SCCs (9).

The nature and number of molecular events leading to malignancy are beginning to be understood. Powerful gene transfer techniques (11, 12) have been developed that have led to the discovery of a class of dominant cellular transforming genes activated in human and rodent tumors. DNAs from these tumors have been found to induce transformation of NIH3T3 fibroblasts upon transfection (13, 14). Many of the transforming genes found so far in diverse animal and human tumors are related to the oncogenes of the RNA tumor viruses. The NIH3T3 focus assay, which has been used most often in the detection of transforming genes, is very sensitive to the detection of mutated members of the ras family (Harvey, Kirsten and N) (15, 16) of proto-oncogenes. The ras family of proto-oncogenes encode for guanine nucleotide binding proteins and in their activated form contain specific point mutations around the 12th and 61st codons. In addition to the detection of activated ras transforming genes, the NIH3T3 focus assay has been useful in detecting other oncogenes including met, neu, raf, erb, B, B-lym and dbl (17–23). In a limited number of studies of ionizing radiation induced animal tumors including mouse lymphomas and rat skin tumors (24), investigators have found activation of the cellular Ki-ras gene. In addition to evidence for Ki-ras activation, a number of radiation induced rat skin and thyroid tumors have been shown to contain genomic amplification and/or rearrangements of the cellular myc gene. In vitro malignant transformation of hamster embryo cells and mouse C3H/10T1/2 cells by X-rays (25, 26) has resulted in the activation of oncogenic sequences not related to the ras family of oncogenes or to any other oncogene characterized so far. Therefore, it was of particular interest to determine whether mouse skin tumors initiated with ionizing radiation contained detectable dominant transforming activity and, if so, determine whether the transforming gene(s) was one of the known cellular oncogenes.

In addition to activation of known oncogenes during multistage carcinogenesis, there is evidence for altered expression of cellular genes at specific stages in the process (27). The altered expression of these genes may play a functional role in establishing or maintaining particular phenotypes associated with tumor progression. One cellular gene that is specifically
overexpressed in malignant SCCs and may play a functional role in tumor cell invasion through the basement membrane is a metalloproteinase gene called stromelysin (28, 29). The stromelysin protease degrades a number of proteins found in the basement membrane including fibronectin, laminin and proteoglycans. We have found overexpression of the stromelysin gene in both chemically and ionizing radiation initiated SCCs when compared to normal epidermis and benign papillomas. An important mechanistic question to be addressed is the molecular explanation for the consistent overexpression of the stromelysin gene in the malignant tumor cells compared to benign tumor cells. This question has lead us to investigate the 5' flanking region of the stromelysin gene for critical cis regulatory elements that may be involved in the transcriptional activation of the gene in ionizing radiation progressed malignant tumor cells.

IONIZING RADIATION AS AN INITIATOR OF MOUSE SKIN TUMORS

Animal studies have shown that ionizing radiation initiates events which are retained in viable cells for a long period of time without any evidence that critical lesion(s) undergo further change until a subsequent event is induced. These studies have suggested that the carcinogenic effect of ionizing radiation can persist for long periods after initial exposure and that the essential event in radiation tumorigenesis may be subsequent to the initial radiation induced lesion. In the mouse skin model, ionizing radiation has been considered a weak carcinogen and extensive studies of the initiating, promoting and progressing potential of this agent have not been pursued until recently. In published work (9, 10), we have studied the initiating potential of a range (7.5 to 22.5 Gy) of 4 Mev X-rays in a two stage model utilizing TPA as a promoting agent in the second stage. We have shown that ionizing radiation acts as a weak initiator in this two stage model system. All groups of animals that were promoted with TPA developed benign papillomas regardless of radiation treatment; however, only those groups of animals that received radiation followed by TPA promotion developed malignant squamous cell carcinomas. In addition to radiation initiated SCCs, we also observed basal cell carcinomas (BCC) in the radiated mice. Our studies were one of the first to report the induction of basal cell carcinomas in mice exposed to ionizing radiation. Basal cell carcinomas occur rarely in experimental animals. The exception is rat skin exposed to carcinogenic doses of ionizing radiation (30). In our experiments the development of basal cell carcinomas was dependent on the dose of ionizing radiation and independent of TPA promotion. In contrast, the induction of squamous cell carcinomas was dependent on the interaction of ionizing radiation and the tumor promoter, TPA. Recently, Ootsuyama and Tanooka (31) have reported the induction of basal cell carcinomas as well as other skin tumors (squamous cell carcinomas and fibrosarcomas) in the mouse using repeated doses of beta irradiation as a complete carcinogen.
IONIZING RADIATION ENHANCES MALIGNANT PROGRESSION OF MOUSE SKIN TUMORS

SCC development in the mouse skin two-stage model using chemical initiators is usually a relatively late and rare event. The frequency of conversion of papillomas to carcinomas is dependent on mouse strain, initiator and promotion protocol. Approximately 10% of chemically initiated papillomas progress to SCCs. Recently, investigators (7) have treated mice that have papillomas with tumor initiators and have observed an increase in SCC formation compared to control mice treated with TPA. These data suggest that progression to malignancy is the result of genetic changes caused by mutagenic and/or clastogenic agents. Since we had shown that ionizing radiation acted as an initiator of skin tumors and radiation is known to be mutagenic and clastogenic, we tested ionizing radiation in the third stage of skin tumor progression (32). CD-1 mice were initiated with N-methyl-N-nitroso-N'-nitro-guanidine (MNNG) followed by biweekly promotion with TPA. After 20 wk of promotion, the animals were treated with acetone, TPA (twice a week for 2 wk) or eight fractions of 1 MeV electrons (1 Gy/fraction over a period of 10 days). The conversion of papillomas to SCCs was 80% for animals treated with ionizing radiation compared to 25% for tumor-bearing animals treated with TPA. Ionizing radiation in the third stage was found to increase the cumulative number of SCCs per group. The lack of an increase in the number of cumulative papillomas per group due to ionizing radiation suggested that the dose and fractionation scheme used in our study enhanced the progression of pre-existing papillomas. The mechanism by which ionizing radiation enhances malignant conversion of benign papillomas is not known. The potential mechanisms could involve further mutagenic or clastogenic events in target papilloma cells or selection of variant pre-existing malignant cells in a large population of benign cells or a combination of the two mechanisms. It is of interest to note that O'Connell and coworkers have recently shown that peroxides (both benzoyl and hydrogen peroxide) can enhance tumor progression (7). Peroxides like ionizing radiation, are capable of generating free radicals. These data are consistent with the hypothesis that free radicals play a role in the progression process.

DETECTION OF DISTINCT TRANSFORMING GENES IN X-RAY INDUCED MOUSE SKIN TUMORS

To examine the role of oncogene activation in radiation-induced mouse skin tumors and address the issue of carcinogen specificity, tumor DNAs were examined for the presence of dominant transforming activity using the NIH3T3 transfection, focus-formation assay (33). We found that eighty two percent of all of the skin tumors DNAs studied were positive in the NIH3T3 focus assay. Transforming activity was observed in all tumor types including papillomas, BCCs, SCCs and pilomatrixomas initiated by ionizing radiation but not in normal epidermis or epidermis of animals initiated with ionizing radiation and promoted with TPA. Dominance of the transforming activity was shown by demonstrating that this
activity could be transferred in a second round of transfection. To study the potential transforming genes transferred from the tumors to the recipient NIH3T3 cells, DNAs from the NIH3T3 transfectant cell lines were analyzed by Southern blot technique for the presence of activated forms of the three cellular ras genes, Harvey, Kirsten, and N. If restriction sites closely linked to the transferred oncogene were destroyed during the transfection process, then DNA prepared from transformed foci would contain novel restriction fragments hybridizing to gene probes of interest. Novel restriction fragments were not observed in any of the NIH3T3 transfectant DNAs examined, nor were any of the endogenous restriction fragments amplified which is often seen when an activated oncogene is transferred to the NIH3T3 genome. These data indicated that the putative transforming genes were not members of the ras gene family. No evidence was obtained for the activation of five other oncogenes that have been detected using the NIH3T3 focus assay. These oncogenes included B-lym, erbB, met, neu, and raf. Further characterization of the dominant transforming genes in the radiation-induced mouse skin tumors was carried out by studying the effects of restriction enzyme digestions on the dominant transforming activity in the transfectant DNAs. The results of mapping of four transfectant DNAs derived from four different SCCs with four different enzymes indicated three different mapping patterns. Our results 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 both Borek et al. (25) and Krolewski and Little (26). Their findings showed that in vitro malignant transformation of mammalian cells (hamster embryo cells and mouse C3H 10T½) by a single direct exposure to X-irradiation resulted in the activation of oncogenic sequences that did not represent activated forms of the ras gene family which have been implicated in chemical transformation of C3H 10T½ cells. In the mouse skin model, when chemical initiating agents are used, the nature and frequency of the activated oncogene is dependent upon the initiating agent used but not upon the tumor promoter (34–36). The ras family of proto-oncogenes is activated by point mutations and the chemical agents or their 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. Since we obtained evidence of multiple transforming genes in the same histology of skin tumor (SCC), our data suggest that oncogene activation in these radiation initiated tumors did not result from a direct effect of ionizing radiation.

ALTERED GENE EXPRESSION IN A X-IRRADIATION DERIVED MALIGNANT EPIDERMAL CELL LINE

Since we observed in the mouse skin that ionizing radiation enhances the progression of chemically initiated papillomas to malignant SCCs we decided to develop a cell culture model to study radiation induced malignant progression. A squamous papilloma producing cell line called 308 (37) which has an activated Harvey-ras oncogene has been used to model the benign tumor state. The 308 cells were irradiated with 10 Gy of x-rays and surviving cell
clones were assessed for their ability to form SCCs in athymic nude mice. One malignantly progressed clone called 308 10 Gy 5 has been further characterized in terms of gene expression. One gene for which we have evidence supporting its role in malignant progression and in particular tumor cell invasion through basement membrane is the stromelysin gene. This collagenase gene encodes for a protease that degrades proteins found in basement membrane. We have hypothesized that the overexpression of the stromelysin gene plays a functional role in malignant progression. Therefore, we have measured the steady state levels of stromelysin message in both parental 308 cells and the malignant variant cell line, 308 10 Gy 5. The variant cells showed measurable expression of the stromelysin gene but in the parental 308 cells there was no detectable expression. A potential reason for the upregulated expression of the stromelysin gene in the 308 10 Gy 5 variant cells could be alterations in either the cis regulatory elements of the gene or changes in the levels of specific trans regulatory proteins. To begin to study the cis and trans regulation of the gene we have cloned approximately 1 kb of 5' flanking region of the mouse stromelysin gene. 850 bp of the promoter region of the gene were cloned into a chloramphenicol (CAT) reporter expression vector. Transient transfection of the mouse stromelysin promoter regulating the CAT reporter gene was carried out using the parental 308 cells and the 308 10 Gy 5 malignant variant cells lines. Constitutive expression of the CAT gene was found using the stromelysin gene promoter in the 308 10 Gy 5 variant cells but no constitutive activity of the promoter was found in the parental 308 cells. These results indicated that there was a cis element in the 850 bp of the mouse stromelysin 5' flanking region that was responsible for the constitutive overexpression of the stromelysin gene in the 308 10 Gy 5 malignant variant cell line compared to the benign parental 308 cells. The exact identity of this cis element is presently being pursued. Sequencing of the 5' flanking region of the stromelysin gene has revealed the presence of a number of consensus cis regulatory elements including a TPA responsive element (TRE) that binds the AP1 transactivating complex. We therefore investigated whether there was upregulated expression of other cellular genes known to contain TREs in the malignant variant cells compared to the parental 308 cells. We found evidence for upregulated expression of the metallothionein IIA, c-fos and c-jun genes in the 308 10 Gy 5 cells when compared to the parental 308 cells. All three of these genes are known to contain TREs.

CONCLUSION

The multistage model of mouse skin carcinogenesis has been a very useful model of carcinogenesis in which both biological and molecular events related to tumorigenesis have been studied. This model has been subdivided into at least three operational stages (ie. initiation, promotion and progression). Initiation is thought to involve permanent genetic alterations, promotion may involve clonal expansion of initiated cells to give rise to a benign tumor and progression may result from multiple genomic alterations that bring about the phenotypic changes seen in malignancy. Chemical carcinogens have been shown to be very
effective in their ability to initiate the process of carcinogenesis. In contrast, ionizing radiation was found to be a relatively weak initiator of malignant squamous cell carcinomas. In addition, a unique histology of skin tumor observed in mice treated with ionizing radiation was basal cell carcinoma, a tumor not normally observed with chemical carcinogens in the skin of mice. Besides demonstrating a weak initiating effect of ionizing radiation, we have shown that 1 MeV electrons in a fractionated dose were effective in enhancing the conversion of chemically initiated benign papillomas to malignant squamous cell carcinomas.

The biological potential of ionizing radiation as a carcinogen in this mouse skin system and the known genomic alterations induced by ionizing radiation tend to suggest that ionizing radiation acts as a weak initiating agent because point mutations are not efficiently induced by this type of radiation. Instead we have found that ionizing radiation functions more efficiently in a third stage of tumor progression which could involve larger genomic alterations more efficiently induced by ionizing radiation. In support of our finding are data from O'Connell's laboratory (7) showing that free radical generating, chemical peroxides, are also effective in enhancing malignant conversion of benign papillomas. These data are consistent with the hypothesis that free radicals play a role in malignant progression.

Our data related to transforming genes in radiation initiated mouse skin tumors suggest that the target gene(s) for oncogenic activation are different for chemical carcinogens and ionizing radiation. Support for our finding has been provided by other researchers investigating in vitro transformation of mouse C3H 10T½ cells by chemicals and radiation. It is perhaps not surprising that activation of the Harvey ras oncogene was not identified in radiation-initiated mouse skin tumors as was consistently observed with chemically initiated mouse skin tumors. The ras family of oncogenes is activated by point mutations and 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. Our finding of at least three different transforming genes in four radiation initiated malignant skin tumors supports the hypothesis that direct activation of transforming genes may not be occurring in radiation carcinogenesis. The presence of multiple transforming genes in these radiation initiated tumors is likely to result from secondary DNA damage due to ionizing radiation direct effects on some cellular genes that regulate DNA replication or genomic stability.

Ionizing radiation has been shown to be effective in the third stage of skin tumor progression. This has been demonstrated both in the whole animal as well as in cultured benign papilloma cells. Though the underlying genetic alterations responsible for progression from a benign to malignant state are not known, we have observed the upregulated expression of a cellular gene that encodes for a secreted metalloproteinase, stromelysin, that encodes for a protease that degrades basement membrane. We have hypothesized that the overexpression of this gene product plays a functional role in tumor cell invasion. We have demonstrated that the overexpression of the stromelysin gene in a radiation induced malignant variant cell line could be mediated at the transcriptional level through a certain cis regulatory element(s) we know is within 850 bp of the transcriptional start site. A candidate cis element that may be involved in this overexpression is a TRE (TPA responsive
element). We know that a TRE is in 5' flanking region of the mouse stromelysin gene. In support of the potential role of the TRE in the regulated overexpression of the stromelysin gene is the fact that we found coordinate overexpression of other cellular genes that are known to contain TREGs in the radiation induced malignant variant cell line. Perhaps the altered expression of the genes with TREGs is mediated through constitutive qualitative or quantitative changes in the AP1 transactivating complex known to transactivate the TRE. How ionizing radiation might mediate such a constitutive effect on AP1 activity is not clear but this possibility is being explored.

REFERENCES


