Irradiated Mammary Gland Stroma Promotes the Expression of Tumorigenic Potential by Unirradiated Epithelial Cells

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ABSTRACT

We have shown that ionizing radiation, a known carcinogen of human breast, elicits rapid, persistent, and global changes in the mammary microenvironment as evidenced by altered extracellular matrix composition and growth factor activities. To address whether these events contribute to radiogenic carcinogenesis, we evaluated the effect of irradiated mammary stroma on the neoplastic potential of COMMA-D mammary epithelial cells. Although COMMA-D cells harbor mutations in both alleles of p53, they are nontumorigenic when injected s.c. into syngeneic hosts. Unirradiated COMMA-D cells transplanted to mammary fat pads cleared previously of epithelia preferentially formed tumors in irradiated hosts. Tumor incidence at 6 weeks was 81% ± 12 SE when animals were irradiated with 4 Gy, 3 days prior to transplantation, compared with 19% ± 2 SE (P < 0.005) in sham-irradiated hosts. This effect was evident when cells were transplanted 1 to 14 days after irradiation. Furthermore, tumors were significantly larger (243.1 ± 61.3 mm^3 versus 30.8 ± 8.7 mm^3) and arose more quickly (100% by 6 weeks versus 39% over 10 weeks in sham hosts) in fat pads in irradiated hosts. The contribution of local versus systemic effects was evaluated using hemibody (I versus right) irradiation; tumors formed only in fat pads on the irradiated side. These data indicate that radiation-induced changes in the stromal microenvironment can contribute to neoplastic progression in vivo. Disruption of solid tissue interactions is a heretofore unrecognized activity of ionizing radiation as a carcinogen.

INTRODUCTION

The multistep model of carcinogenesis defines cancer initiation as genomic change and promotion as the series of events leading to proliferation of initiated cells. Agents that promote carcinogenesis are generally thought to increase the probability that a cell will acquire additional mutations necessary for neoplastic progression. Quantitative studies in rodent models demonstrated that the number of cells initiated, as evidenced by morphological transformation in culture after either physical or chemical carcinogen exposure, far exceeds the number of tumors that develop in vivo (1, 2). Because initiation appears to be a frequent event (3), the factors that facilitate expression of tumorigenic potential are critical in determining cancer frequency but are still poorly understood.

The contribution of stroma to early events in carcinogenesis has recently begun to be appreciated. Whereas it is well-recognized that tumors can recruit cells (e.g., endothelial cells) and induce changes in the stroma that are conducive to their growth (4, 5), it has also been suggested that disruption of stromal/epithelial interactions may provide a stimulus for initiated cells to move further down the neoplastic pathway (6, 7). Specialized microenvironments, composed of insoluble ECM and soluble growth factors, mediate epithelial-stromal interactions and play a pivotal role in normal tissue development and function (8, 9). Such interactions can efficiently suppress the expression of the neoplastic phenotype (3, 10, 11). Conversely, abnormal stromal/epithelial interactions have been shown to enhance the ability of cells to express the neoplastic phenotype (12, 13). Transgenic manipulation of the microenvironment, rather than the target cell, can also stimulate tumorigenesis (14). Recent studies in chronic myelogenous leukemia and in human mammary cancer cells indicate that reestablishing appropriate interactions with the ECM can reverse tumorigenesis and neoplastic behavior, even in the presence of grossly abnormal genetic damage (15, 16).

Irradiating radiation is a complete carcinogen, able both to initiate and promote neoplastic progression (17), and is a known carcinogen of human breast (18–21) and rodent mammary glands (22–24). Our previous studies have demonstrated that radiation exposure elicits rapid and persistent global remodeling of the mammary gland ECM (25). Because cell-ECM interactions are pivotal in mammary differentiation and growth control (26, 27), we postulated that the radiation-induced mammary microenvironment may contribute to radiation carcinogenesis by disrupting cell interactions in a manner that is conducive to expression of neoplastic potential (28).

To test the above hypothesis, we transplanted epithelial-free mammary stroma with the functionally normal COMMA-D mammary epithelial cell line. The COMMA-D mammary epithelial cell line arose spontaneously from a primary culture of epithelial cells from mammary glands of pregnant BALB/c mice and is nontumorigenic at early passages (29). It was shown subsequently to contain two mutations in p53 characterized as substitution of Trp for Cys at codon 138 and deletion of the first 21 nucleotides of exon 5 (30), which result in nuclear accumulation (31). The mutations in p53 in these cells suggest that the population may be genomically unstable and susceptible to neoplastic transformation (30).

We show here that COMMA-D cells transplanted to cleared mammary glands in irradiated hosts gave rise to significantly more tumors that arose more quickly and grew larger than in unirradiated animals. Hemibody irradiation supports the conclusion that radiation effects on mammary microenvironment is critical because tumors arose only when COMMA-D cells were transplanted to the irradiated side of animals. These data demonstrate that radiation effects on stroma facilitate expression of neoplastic potential in the absence of exogenously induced mutagenic events in mammary epithelial cells.

MATERIALS AND METHODS

Animals. Female BALB/c mice were obtained from Simonson (Gilroy, CA) and housed five per cage with chow and water ad libitum in a temperature- and light-controlled facility. When necessary, mice were anesthetized using xylazine (40 mg/kg) and ketamine (25 mg/kg) injected i.m. Carbon dioxide inhalation was used to kill the animals in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and institutional review and approval. The fourth inguinal mammary glands were removed for histology and whole mounts.

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Cell Culture. COMMA-D mouse mammary epithelial cells originating from midpregnant BALB/c mice (29) were obtained at passage 11 from Dr. Peggy Neville (University of Colorado, Denver, CO) and were expanded and frozen at passage 13. Cells were grown routinely in 1:1 F12:DMEM supplemented with 2% fetal bovine serum, epidermal growth factor (5 ng/ml), insulin, transferrin, and selenium on tissue culture plastic in a 5% CO₂ humidified incubator at 37°C and passaged twice weekly at <90% confluency. Cells were used between passages 15 and 24. Differential trypsinization was used to enrich for morphological variants at passage 22 by collecting cells released in the first 3 min of trypsinization (CD-3T), adding trypsin for an additional 2 min (discarded), followed by an additional 5 min of trypsinization to release the remaining cells (CD-5R). Each subpopulation was expanded one passage, and aliquots were frozen for subsequent characterization. Cells were trypsinized, counted, and resuspended in serum-free medium or PBS immediately before transplantation.

Transplantation Assay. The female mammary gland is unique among all glands in that the epithelium develops postnatally from a rudiment that can be removed from the inguinal glands at ~3 weeks of age (32). Surgical removal of the parenchyma results in a gland-free mammary fat pad, referred to as a CFP, suitable for receiving donor tissue at the time of clearing or later (32). Transplantation of normal mammary epithelial cells produces ductal outgrowths that fill the fat pad and are nearly indistinguishable in whole mounts or histologically from intact gland (32). Anesthetized 3-week-old mice were opened s.c. at the anterior midline and along each flank to reveal the inguinal mammary gland. The epithelium was destroyed by cauterying the connection to the 5th gland, and removing the tissue from the nipple to just above the lymph node, leaving approximately two-thirds of the fat pad. The procedure was repeated on the opposite side, and the skin was closed with wound clips.

Mammary glands were cleared of epithelia at 3 weeks of age. The mice were irradiated and transplanted at 10–12 weeks of age. The CFPs were exposed from the rostral midline to avoid the scar tissue formed by clearing. Known numbers of COMMA-D cells were injected using a Hamilton syringe into the fat pad in a volume of 10 μl. Animals were monitored weekly for gross changes and sacrificed at 6–10 weeks as indicated, and the inguinal glands were removed for analysis. Glands were fixed in 100% ethanol:glacial acetic acid (3:1) for 24 h, followed by alum carmine, according to published methods (33). Tumor sizes were determined by caliper measurements of width, thickness, and length in the whole mounts, and the approximate tumor volume was calculated as the product of these. Selected tissues were fresh frozen in OCT (Miles Associates, St. Louis, MO).

Irradiation. Unanesthetized mice were whole-body irradiated in perfluorated Plexiglas tubes using 60Co γ-radiation at a dose rate of 0.32 Gy/min to a total dose of 4 Gy. Dosimetry was determined using an Victoreen ionization chamber. Anesthetized animals were used for hemibody irradiation (4 Gy) by shielding the right lateral half with lead collimators. Control animals were sham-irradiated with or without anesthesia, as indicated.

Immunofluorescence. Cells were seeded in 24-well plates in 1 ml of 0.5% serum-containing medium. After reaching confluence, cells were washed with PBS and fixed with 500 μl of methanol for 10 min at ~20°C for vimentin and keratin staining and in methanol overnight at 4°C for p53 immunostaining. Fixed cells were stored in PBS at 4°C before immunostaining. Cryosections (5 μm) were fixed with 2% buffered paraformaldehyde for 10 min for p53 immunostaining and 4% buffered paraformaldehyde for 10 min for vimentin and keratin immunostaining.

Polyclonal rabbit antibodies against p53 protein (CM5; Vector Laboratories, Burlingame, CA) were used at 1:500 (0.5 μg/ml). Monoclonal mouse antibodies against vimentin (clone VIM 13.2; Sigma) were used at 1:200 dilution (10 μg/ml), and polyclonal guinea pig antibodies to keratin (Sigma) were used at 1:100 dilution. Nonspecific reactivity was blocked with the supernatant of 0.5% casein stirred for 1 h in PBS. Specimens were incubated with 50 μl of primary antibody overnight (18–20 h) at 4°C. Vimentin and pan-keratin primary antibodies were incubated together, followed by sequential incubation with fluorochrome-labeled secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI at 0.5 μg/ml (Sigma) during the last wash. Specimens were mounted with Vectashield mounting medium (Vector Laboratories) and stored at ~20°C prior to viewing. Whole mounts were embedded in paraffin and sectioned at 5 μm for standard histology staining with H&E.

Sections from whole mounts were incubated with p53 antibodies as above and detected with alkaline phosphatase-linked secondary antibody. Image Acquisition and Processing. Fluorescence microscopy of double or triple fluorescence-stained sections were imaged using a Zeiss Axiosvert equipped with epifluorescence and multiband pass filter and differential wave-length filter wheel. Images were acquired using a scientific-grade 12-bit charged coupled device (KAF-1400, 1317 × 1035 6.8-μm square pixels) camera (Xillix, Vancouver, Canada). The images were captured so that intensities for a given experiment fell within the 12-bit linear range. Relative intensity was maintained when constructing figures by scaling the data set to a common 8-bit scale using Scilimage (TNO, Delft, the Netherlands).

Statistical Analysis. Tumor incidence was evaluated using 95% confidence intervals and the z test of proportion for data derived from a single experiment or two-tailed t test for data from multiple experiments (SigmaStat, Santa Rosa, CA). The significance of differences between tumor size for sham-irradiated control and irradiated animals were determined using two-tailed Mann-Whitney rank sum test (34).

RESULTS

COMMA-D Cells Retain Mammary Developmental Potential and Are Nontumorigenic. COMMA-D cells are functionally intact in that they can be induced by lactogenic hormones to synthesize a number of milk proteins, including β-casein (35). We confirmed that the cell line also retains the capacity to produce ductal outgrowths in vivo when early-passage cells are transplanted to CFPs (29) and undergo morphogenic reorganization into acini when cultured on a complex basement membrane-type matrix (25). When transplanted into CFPs of 3-week-old mice, COMMA-D cells form a simple mammary ductal outgrowth that fills 25–100% of the stroma. These outgrowths were confirmed as originating from COMMA-D cells by nuclear p53 immunoreactivity, which was absent from stromal cells (not shown). Tumors were not observed when COMMA-D cells were transplanted at the time of clearing in 3-week-old animals or when injected s.c. into adult syngeneic hosts (0.5, 1, or 2 million) over a period of 8 weeks (n = 6, not shown).

COMMA-D Cells Transplanted to CFPs of Irradiated Hosts Establish Tumors Rapidly. Our previous studies indicated that ionizing radiation leads to global remodeling of the ECM and induces activity of potent modulators of epithelial behavior (25, 36, 37). Individually irradiated mammary glands exhibit the same microenvironment changes as those from whole-body-irradiated animals, indicating that these effects are mediated by local factors. CFPs from irradiated mice show similar remodeling of the microenvironment as that observed in intact mammary glands (not shown).

We tested whether the irradiated mammary microenvironment could modulate the neoplastic potential of COMMA-D mammary epithelial cells. Different numbers of unirradiated COMMA-D cells were transplanted to CFPs in adult mice that were sham-irradiated or that received 4 Gy 60Co γ-radiation 3 days prior to transplantation (Fig. 1). A single tumor formed in sham-irradiated CFPs transplanted with 2 × 10⁶ cells (n = 6); no tumors were observed when fewer cells were injected. However, tumors arose in irradiated CFPs as a function of cell number, even when transplanted with as few as 2.5 × 10⁵ cells. Every CFP (100%) from irradiated hosts contained tumors when injected with 2 × 10⁶ unirradiated COMMA-D cells. In four independent experiments (n = 36 injected CFPs), tumor incidence was significantly (P < 0.005, two-tailed t test) greater in irradiated CFPs (30 of 36) versus sham-irradiated (8 of 36). The percentage of CFP-bearing tumors averaged 19% ± 2 SE in sham-irradiated hosts and 81% ± 12 SE in hosts irradiated with 4 Gy, 3 days prior to transplantation.

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4 E. J. Ehrhart and M. H. Barcellos-Hoff, unpublished data.
Ductal outgrowths were obtained at low frequency (~25%) in adult mice, but the frequency and character were similar in irradiated and sham-irradiated CFPs. In addition, we found that the transplantation take of normal mammary tissue fragments to sham and irradiated hosts was equivalent. Thus, the ability of cells to survive and/or grow in mammary glands in both sham and irradiated hosts was similar. Some tumors were found in association with otherwise normal-appearing ductal outgrowths (Fig. 2A), but most were isolated.

Nuclear accumulation of p53 was evident in ductal outgrowths and tumors and was lacking from cells in adjacent stroma (Fig. 2B). Tumors were both vimentin and keratin positive and exhibited nuclear p53, indicative of their origin from COMMA-D cells (Fig. 2C). Note that p53 immunostaining was heterogeneous but more evident using the more sensitive immunofluorescence detection. This heterogeneity is also evident in culture (Fig. 4).

The persistence of radiation effects was determined by transplanting COMMA-D cells to CFPs as a function of time after exposure to 4 Gy whole-body 60Co γ-radiation exposure (Fig. 3). All irradiated animals showed a significant (P < 0.05) increase in tumor incidence, ranging from 100% at 3 day to 58% at 14 days after irradiation compared with 25% in the sham-irradiated host. The peak occurred when irradiated animals were transplanted at 3 days after irradiation, at which time tumors arose in 100% of glands in irradiated hosts. Furthermore, the mean size of tumors from irradiated animals was 243 mm³ ± 61 SD compared with 31 mm³ ± 9 SD in the few tumors that arose in sham-irradiated hosts. The mean size of tumors in irradiated animals at all times after irradiation were significantly (P < 0.05; Mann-Whitney rank sum test) larger than the size of tumors in sham-irradiated control animals.

To test whether tumor formation was accelerated in the irradiated CFPs, we evaluated tumor incidence as a function of time after transplantation. Tumors arose too quickly in irradiated mammary glands (100% by 6 weeks) to permit extending their observation period. The overall tumor incidence in sham-irradiated hosts over a period of 10 weeks was 39% of transplanted CFPs examined. Tumor incidence decreased from 6 to 10 weeks in sham-irradiated hosts, suggesting that these small tumors may regress. Thus, we had been able to carry the irradiated mice to 10 weeks, the difference between the two groups would be even more dramatic.

COMMA-D Parental Heterogeneity Gives Rise to Subpopulations That Are Also Preferentially Tumorigenic in Irradiated Hosts. Clonal COMMA-D subpopulations have been shown to retain the capacity to produce ductal outgrowths and to respond to hormone stimulation (38), suggesting that some COMMA-D cells have multipotent characteristics of putative stem cells. The multipotent nature of these cells is also suggested by the observation that COMMA-D cell cultures contain multiple morphologically distinct cells consisting of flat, polygonal cells that form contact-inhibited monolayer islands and spindle-shaped cells that form ridges that surround the islands. This
heterogeneity is maintained by routine passaging at 80–90% confluency. These cells are p53 positive and exhibit various levels of keratin and vimentin immunoreactivity (Fig. 4A and E). Keratin is observed predominantly in polygonal cells, whereas vimentin expression is less prominent and is associated with the ridges of spindle cells.

The possibility that these distinct cell types have different tumorigenic potentials or are preferentially selected during growth in vivo was addressed by testing their respective behaviors in CFPs. Morphologically distinct subpopulations were enriched from the parent population by differential trypsinization. Trypsinization releases spindle-shaped cells in the first 3 min (designated CD-3T), whereas predominantly polygonal cells remain after 5 min of trypsinization (designated CD-5R). Both cell types exhibited p53 immunoreactivity in culture (Fig. 4, E–G). The CD-3T subpopulation was primarily vimentin positive and keratin negative and had large nuclei (Fig. 4, B and F). The CD-5R subpopulation contained keratin-immunoreactive cells, with little expression of vimentin, and had small nuclei (Fig. 4, C and G).

Both the CD-3T and CD-5R subpopulations were significantly (P < 0.05) more tumorigenic in irradiated hosts when transplanted to sham- versus irradiated-hosts (Fig. 5). However, the CD-5R subpopulation produced fewer tumors (4 of 12) than the parent in irradiated CFPs and did not give rise to any tumors in the sham-host. The tumors from the CD-5R were also considerably smaller than either the parent or the CD-3T subpopulation (Fig. 5B). In contrast, the CD-3T subpopulation was more efficient (12 of 12) in generating tumors than the parent population (8 of 12) in this experiment. Furthermore, unlike the parent COMMA-D population, the size of tumors from the 3T-CD subpopulation were similar in the sham-irradiated CFPs and irradiated CFPs. Thus, the size of the tumors is influenced by both the nature of the microenvironment and of the epithelial population.

COMMA-D Cells Are Tumorigenic Only in the Irradiated CFPs of Hemibody-irradiated Mice. Radiation might promote neoplastic behavior by causing aberrant immune or endocrine function. Partial (left versus right) body 60Co γ-irradiation of anesthetized mice was used to test whether systemic factors from the irradiated host contributed to tumor promotion in irradiated CFPs. No tumors were found in nonirradiated CFPs (n = 8), whereas three tumors were
observed in irradiated CFPs ($P = 0.05$). To determine whether the low incidence of tumor formation was attributable to the systemic effects of anesthesia necessary for hemibody irradiation, we asked whether anesthesia compromised tumor formation. Anesthetized, whole-body-irradiated mice formed tumors in two of eight transplanted CFPs, whereas unanesthetized, whole-body-irradiated mice in this experiment formed seven tumors in eight CFPs. Thus, the low incidence of tumors in the partial body protocol compared with whole-body exposures appears to be attributable to the use of general anesthesia; tumor incidence was restricted to CFPs on the irradiated side, indicating that local tissue effects were dominant over the systemic consequences of irradiation.

**DISCUSSION**

Although the stroma has generally been considered a silent bystander during epithelial carcinogenesis, the concept that the microenvironment is central to maintenance of cellular function and tissue integrity provides the rationale for the idea that its disruption can contribute to neoplasia (8). Indeed, it has been suggested that cancer may be a physiological response to an abnormal environment (6, 14, 39, 40), and evidence is accumulating that support such hypotheses (41). Most studies of radiogenic carcinogenesis emphasize radiation-induced DNA damage because mutations are believed to initiate carcinogenesis. However, low doses of ionizing radiation after the composition of tissue microenvironment by rapidly affecting cytokine production and activities (36, 37), ECM composition (25, 37, 42), and expression of receptors that mediate cell-cell interactions (43–45).

The studies reported here address the question of whether radiation-induced changes in the composition of the mammary microenvironment promote the expression of neoplastic potential. We showed that COMMA-D mammary epithelial cells, which retain normal developmental capacity, were nontumorigenic by standard assays (lack of tumor formation when injected s.c. or in juvenile CFPs). Small, infrequent (19%) tumors were obtained when COMMA-D cells were transplanted to adult CFPs, suggesting that the physiological status of the host can modulate COMMA-D neoplastic potential. Tumorigenic efficiency increased to 100% when unirradiated COMMA-D cells were transplanted to CFPs in irradiated hosts. Furthermore, tumor size also substantially increased in irradiated CFPs. The effect of radiation on enhanced tumorigenesis persisted for at least 14 days after a single radiation exposure. We concluded that this was attributable to radiation-induced changes in the mammary stroma by comparing the irradiated and nonirradiated CFPs after hemibody irradiation; tumors formed only in the CFPs from the irradiated side. Thus, radiation exposure altered the mammary microenvironment in a manner that promotes the neoplastic behavior of these mammary epithelial cells, which harbor defective p53.

Although clonal, COMMA-D cells exhibit morphological and phenotypic diversity in culture (30, 38). We therefore determined some of the characteristics of the tumors arising from COMMA-D cells and found that tumors maintained cytokeratin immunoreactivity whether they arose in normal or irradiated CFPs. We then used selective trypsinization to isolate the CD-3T vimentin-positive versus the CD-5R keratin-positive subpopulations and asked whether these subpopulations showed distinctly different characteristics from the parent. CD-5R were less tumorigenic and considerably smaller than the parent cell line. In contrast, the CD-3T subpopulations were somewhat more efficient than the parent cell line in establishing tumors, and unlike the parent, were as large in the sham-irradiated host as in the irradiated host.

Our previous studies showed that irradiated mammary gland undergoes rapid remodeling of the microenvironment characterized by changes in ECM and activation of latent TGF-$\beta$ (25, 36). We tested whether these events were functionally related by treating animals with TGF-$\beta$ neutralizing antibodies prior to irradiation (37). TGF-$\beta$ panspecific neutralizing antibody administered shortly before irradiation inhibited collagen III staining in the adipose stroma at 24 h in an antibody dose-dependent manner and blocked collagen III through 7 days after irradiation. Quantitative image analysis demonstrated that exposure to radiation doses of as little as 0.1 Gy elicited a significant increase in TGF-$\beta$ immunoreactivity, which showed a linear dose response after exposure to 0.1–5 Gy without an apparent threshold of the mammary epithelium. Qualitative evaluation of the reciprocal pattern of latency-associated peptide and collagen III immunoreactivity in the adipose stroma, in contrast, demonstrated a threshold of 0.5 Gy. These data provide functional confirmation of the hypothesis that radiation induces TGF-$\beta$ activation and implicate TGF-$\beta$ as a mediator of tissue response to ionizing radiation.

TGF-$\beta$ orchestrates responses of multiple cell types and has emerged as a key coordinator of tissue response to damage during wound healing, inflammation, and development. Aspects of radiation remodeling parallel those associated with dermal wound healing (46). Ionizing radiation effects on the tissue microenvironment that foster neoplastic behavior may be similar to those observed by other agents that elicit an activated stroma. Experimental animal models have demonstrated that carcinogenesis is enhanced by the activated stroma

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*Fig. 5. Tumor formation by COMMA-D parent cells and subpopulations, CD-3T and CD-5R, transplanted into CFPs in sham-irradiated hosts (▼) or hosts irradiated 3 days before transplantation with 4 Gy (●). A, tumor incidence was significantly ($P < 0.05$) greater in irradiated versus sham hosts for all three populations. B, tumor size varied as a function of cell population and of irradiation status.*
induced by wounding (12), overexpression of platelet-derived growth factor (5), or misregulation of stromelysin (41, 47).

Although the particular aspect of the irradiated mammary microenvironment that is stimulating COMMA-D tumorigenic conversion is unknown, the ability of radiation to induce TGF-β activation may indeed play a role (36, 48). We have argued that the role of TGF-β in cancer appears paradoxical in that normal epithelial cells are profoundly sensitive to TGF-β growth inhibition, whereas cancer cells are generally TGF-β resistant (49) because conversion during tumor progression is a critical juncture in establishing malignant behavior of certain epithelia, particularly skin and breast (50). TGF-β has been identified as a cancer-promoting agent in wounds (13, 51, 52), and recent studies have implicated cyclosporin-induced TGF-β in the increased frequency of neoplasms after immunosuppressive therapy (53). TGF-β also mediates phenotypic conversion of epithelial cells to vimentin-positive spindle cells (54). Such a transition is observed in a transgenic keratinocyte model overexpressing TGF-β activity in which the frequency of benign papillomas is suppressed after chemical carcinogenesis, but progression to more malignant spindle cell carcinomas is stimulated (55). In the SCp2 cell line, a subclone of late-passage COMMA-D cells (56), TGF-β treatment stimulates the transition from nontumorigenic keratin-positive cells to tumorigenic, vimentin-positive cells.8 Studies to determine whether the carcinogenesis-promoting effect of radiation on stroma is attributable to TGF-β activation are under way.

Our hemibody irradiation data support the conclusion that radiation alters the local tissue microenvironment in a way that compromises the restraints imposed by normal stroma on initiated epithelial cells. A role for stroma early in neoplastic progression has also been suggested in hematopoietic malignancies, which have been proposed to result from misregulation of adhesive properties by diseased or genetically aberrant stroma (57). Conversely, the therapeutic benefit of IFN-α in chronic myeloid leukemia has been shown recently to be attributable in part to the reestablishment of cell adhesion signals (15). Greenberger et al. (58) proposed a model of indirect γ-irradiation leukemogenesis based on cocultures of heavily irradiated bone marrow stromal cell lines that selectively bound macrophage-colony-stimulating factor receptor-positive unirradiated hematopoietic progenitor cells, resulting in selection of tumorigenic subclones. Additional evidence that radiation effects on stroma alter the behavior of neoplastic cells comes from studies of tumor bed effect, in which stroma that is heavily irradiated prior to tumor transplantation inhibits tumor growth but fosters metastatic behavior (59). Such studies support the conclusion that radiation has global and persistent consequences in terms of stromal function, which in turn can influence the expression of neoplastic potential.

In effect, these experiments demonstrate a bystander-type phenomenon in vivo in which the products of irradiated cells can significantly alter the phenotype of unirradiated cells. On the basis of these data, we propose that radiation-induced microenvironments are evidence of an additional class of carcinogenic action, distinct from those leading to mutations or proliferation (60). Studies in cell culture indicate that the frequency of morphological transformation can be modulated by restrictive conditions that select for preexisting cell variants (61) and that, conversely, normal cells may actively restrain the expression of the transformed cell phenotype (62). In vivo studies by Cha et al. (63) show that mammary tumors with Hras1 mutation genes from N-nitroso-N-methylurea-treated rats arose from cells with preexisting Hras1 mutations that occurred during early development. Thus, although clearly mutagenic in its own right, N-nitroso-N-methylurea exposure led to the expansion and neoplastic progression of Hras1-mutation containing populations. In our studies, radiation did not directly induce additional mutagenic events because the epithelial cells were unirradiated. We propose that a further action of carcinogens, such as ionizing radiation, is to modify paracrine interactions between the stroma and epithelium in a manner that affects the frequency with which previously initiated cells progress (46). Carcinogen-induced microenvironments are not necessarily mutagenic or mitogenic per se. Rather, changes in the microenvironment may promote neoplastic behavior by disrupting normal cell functions that are regulated through cell-cell contact, cell-ECM interactions, and growth factor signaling. Thus, if ionizing radiation induces a microenvironment that modifies restrictive interactions, then it may promote malignant phenotype in a manner that is functionally equivalent to the acquisition of additional mutations in the initiated cell. Alternatively, the microenvironment elicited by carcinogen exposure could create novel selective pressures that would affect the features of a developing tumor. Disruption of solid tissue interactions is a heretofore unrecognized activity of radiation as a carcinogen and a novel avenue by which to explore new strategies for intervening in the neoplastic process.

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