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These studies address the question of how abnormal stromal-epithelial interactions affect the progression of cancer cells. Our studies in mouse mammary gland reveal that ionizing radiation, a known human breast carcinogen, elicits rapid and persistent global changes in the tissue microenvironment. If the microenvironments induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of alterations in stromal and epithelial phenotypes. The current studies are intended to test the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to genomic damage or proliferative advantage*. The long-term goal of this research is to determine whether definition of carcinogen-induced microenvironments predicts neoplastic features or frequency. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be suitable for therapeutic intervention, which in turn could provide the mean to modify cancer progression.

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FOREWORD

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INTRODUCTION

We have proposed the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to mutation or proliferative advantage.* Carcinogen-induced microenvironments are postulated to increase the number or susceptibility of epithelial cells to transformation, exert a selective force on initiated cells and/or are conducive to progression. If the microenvironment induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of carcinogen-induced phenotypic change. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be amenable to modulation, which in turn could provide the means to modify cancer progression. The proposed studies are intended to obtain further evidence for this hypothesis.

We have studied the effects of a known breast carcinogen, ionizing radiation, on the microenvironment of the mouse mammary gland. We have showed that mouse mammary gland extracellular matrix undergoes rapid and global remodeling that includes the novel expression of tenascin and collagen type III. This remodeling is mediated by the activation of the multipotent cytokine, transforming growth factor-β1 (TGF-β), a potent regulator of both epithelial and stromal function. We have shown that activation can be detected at doses as low as 0.1 Gy and that blocking TGF-β with neutralizing antibodies inhibits radiation-induced extracellular matrix remodeling, providing functional confirmation of TGF-β activity. Based on these studies, we concluded that exposure to a carcinogen such as radiation can elicit persistent changes in gene expression by non-initiated cells.

By creating chimeric mammary glands consisting of normal or irradiated mammary epithelium in normal or irradiated stroma, we found that the irradiated stroma impedes epithelial maturation. The first aim of the present grant is to test whether radiation-induced TGF-β activity regulates this phenotype. The effect of the irradiated stroma may relate to the well-documented age dependence of radiogenic breast cancer. For example, if radiation-induced microenvironment delays the development of the gland in differentiating past a critical check point, then the size or sensitivity of the carcinogen-susceptible population may be increased. Alternatively, radiation-induced TGF-β may be a selective force that allows expansion of initiated cells resistant to TGF-β. To test whether preneoplastic cells progress more readily in an abnormal stroma, we propose in aim two to create chimeric glands consisting of preneoplastic epithelium in normal versus irradiated stroma.

We predicted that, given the known age dependence of radiogenic mammary cancer in both mice and women, the character of the microenvironment would change as a function of both radiation exposure and mammary development. Our third objective is to compare the radiation-induced microenvironment of adult and immature mice, with particular attention to the expression and activity of TGF-β. The regulation of TGF-β activation and activity in vivo is not well-understood. We have begun this study by examining the effect of development, hormonal status, and differentiation on TGF- β activation.

Year 3 STUDIES

Aim 1: Determine the role of TGF-β in the inhibition of mammary gland development by irradiated stroma by using neutralizing antibodies to knockout TGF-β activity during outgrowth.

We have demonstrated that remodeling of the irradiated mouse mammary gland microenvironment is mediated in part by TGF-β (Ehrhart, 1997). TGF-β is an important regulator of differentiation, proliferation and extracellular matrix composition. It has been postulated to play both a positive and negative role in cancer development and progression (Roberts et al., 1988), which suggests that determining its physiological regulation and activity in particular tumors may provide interesting targets for therapy (Reiss and Barcellos-Hoff, 1997).

TGF-β is secreted as a latent complex that is unable to bind to TGF-β receptors until the biologically active 24-kD mature TGF-β is released; this activation is considered to be the critical regulatory event for TGF-β function. Radiation exposure elicits rapid and persistent activation of TGF-β *in vivo*. We postulated that aberrant TGF-β activation by ionizing radiation affects mammary gland development and neoplastic progression by perturbing the balance between the stroma and epithelium.

The effects of TGF-β in the mammary gland are complex and not well understood. Prior to examining the effect of developmental status on the response to radiation, we reported the distribution and abundance of active TGF-β in Balb/c mice during normal mammary development last year. Using an immunostaining protocol that preserves endogenous latent TGF-β and antibodies that discriminate between latent and active TGF-β, we determined that in normal adult mammary gland latent TGF-β is abundant but active TGF-β is restricted to epithelial structures. To evaluate the consequences of TGF-β activity during development, we have now examined mammary gland development in TGF-β knockout mice.

TABLE I: Ductal elongation and proliferation in TGF- β +/- mice is increased.

Genotype	% Fatpad filled [a] (n=11)	% PCNA (+ / total) [b]	
		Endbuds	Ducts
Wildtype	27	2.7 (34/1265)	1.7 (19/1091)
Heterozygote	61	8 (117/1460)	4.3 (44/1015)

[a]Wholemounts of mammary glands were scored for ductal filling, without knowledge of genotype, using a steromicroscope. Growth to the distal edge of the lymph node was considered 25% filled.

[b]Areas for counting were selected at low power using DAPI nuclear counterstain to identify ducts and endbuds in duplicate sections of replicate animals. PCNA staining was scored, without knowledge of genotype, by counting positive cells in the fluoroscein image and total epithelial cells in the DAPI image. A total of 12 fields and more than a thousand cells were counted for each condition.

Haploid TGF-β genotype leads to accelerated mammary ductal growth. TGF-β null mutant mice have been very informative in demonstrating that the TGF-β isoforms perform distinct functions in vivo even though they share sequence homology and receptors (Letterio and Roberts, 1996). This specificity may in part be due to distinctive LAP sequences that dictate both localization and susceptibility to activation (Barcellos-Hoff, 1996). Mice in which TGF-β is deleted by targeted gene knockout die at weaning from grossly inflamed tissues (Kulkarni et al., 1993), which thus

precludes analysis of postnatal mammary development. However, TGF-β heterozygotes exhibit significantly compromised TGF-β levels (10-30% of wild type), indicating that endogenous TGF-β regulates, directly or indirectly, its own production and/or stability (Tang et al., 1998). We evaluated TGF-β heterozygotes to determine whether reduction of endogenous TGF-β alters mammary growth. Mammary glands of TGF-β heterozygotes examined during puberty (6 weeks of age) showed a significant increase in mammary gland development (Table 1). Whole mount analysis revealed that duct elongation is 2-fold greater in heterozygotes compared to wild-type littermates. PCNA, a marker of cell cycle status is elevated approximately 3-fold in endbud epithelial cells, and more than 2-fold in ducts. Thus, reduction of endogenous TGF-β expression gave rise to accelerated morphogenesis due in part to increased mammary epithelial proliferation. Surprisingly, mature glands from heterozygote mice were undistinguishable from wild-type mice, in both whole-mount appearance (not shown).

These animals provide a new model in which to test the significance of TGF-β in the irradiated stroma. We have determined that collagen III remodeling is different in the TGF-β heterozygote than its wildtype siblings following radiation exposure. We are currently comparing other aspects of the radiation response with the intent to examine the effect of irradiated host on unirradiated epithelium.

Aim 2: Determine the effect of sham versus irradiated fat pads on the carcinogenic potential of the COMMA1-D mammary epithelial cell line harboring defective p53 genes.

We have submitted a manuscript (summarized below) on our studies testing whether the irradiated stroma affects the neoplastic potential of unirradiated COMMA-D mammary epithelial cells.

COMMA-D cells retain mammary developmental potential and are non-tumorigenic.

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 (Medina et al., 1987). We confirmed that the cell line also retains the capacity to produce ductal outgrowths in vivo when early passage cells are transplanted to CFP (Danielson et al., 1984) and undergo morphogenic reorganization into acini when cultured on a complex basement membrane type matrix (Barcellos-Hoff, 1993). COMMA-D cells form a simple mammary ductal outgrowth when transplanted into CFP of 3 week old mice and were confirmed as originating from COMMA-D cells by nuclear p53 immunoreactivity, which was absent from stromal cells. Tumors were not observed when COMMA-D cells were transplanted at the time of clearing in 3 week old animals or when injected subcutaneously into adult syngenic hosts (0.5, 1 or 2 million) over a period of 8 weeks (n=6, not shown). In addition COMMA-D cells injected subcutaneously in nude mice do not form tumors.

COMMA-D cells transplanted to CFP of irradiated hosts establish tumors rapidly.

Our previous studies indicated that ionizing radiation leads to global remodeling of the extracellular matrix and induces activity of potent modulators of epithelial behavior (Barcellos-Hoff, 1993; Barcellos-Hoff et al., 1994; Ehrhart, 1997). 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 (E.J. Ehrhart and M.H. Barcellos-Hoff, unpublished data). CFP 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 CFP in adult mice that were sham-irradiated or that received 4 Gy Co^{60}-γ radiation 3 days prior to transplantation. A single tumor formed in sham-irradiated CFP transplanted with 2 x 10^6 cells (n=6); no tumors were observed when fewer cells were injected. However, tumors arose in irradiated CFP as a function of cell number, even when transplanted with as few as 2.5 x 10^5 cells. Every CFP (100%) from irradiated hosts contained tumors when injected with 2 x10^6 unirradiated COMMA-D cells. In four independent experiments, tumor incidence was significantly ($P<0.005$, two-tailed t-test) greater in irradiated CFP. Tumor incidence averaged 19% ± 2 S.E.M. in sham irradiated hosts and 81% ± 12 S.E.M. in hosts irradiated with 4 Gy, 3 days prior to transplantation.

Ductal outgrowths were obtained at low frequency (approximately 25%) in adult mice, but the frequency and character were similar in irradiated and sham-irradiated CFP. In addition, we found that the transplantation of normal mammary tissue fragments to sham and irradiated hosts was equivalent (unpublished observations). 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, but most were isolated.

Nuclear accumulation of p53 was evident in ductal outgrowths and tumors and was lacking from cells in adjacent stroma. Tumors were both vimentin and keratin positive and exhibited nuclear p53, indicative of their origin from COMMA-D cells.

The persistence of radiation effects was determined by transplanting COMMA-D cells to CFP as a function of time after exposure to 4 Gy whole body Co^{60}-γ radiation exposure. All irradiated animals showed a significant ($P<0.05$) increase in tumor incidence, ranging from 100% at 3 day to 58% at 14 days post-irradiation compared to 25% in the sham-irradiated host. The peak occurred when irradiated animals were transplanted at 3 days post-irradiation, at which time tumors arose in 100% of glands in irradiated hosts. Furthermore, the mean size of tumors from irradiated animals was 243 mm^3 ± 61 S.D. compared to 31 mm^3 ± 9 S.D. in the few tumors that arose in sham-irradiated hosts. The mean size of tumors in irradiated animals at all times post-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 CFP, we evaluated tumor incidence as a function of time post-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 weeks to 10 weeks in sham-irradiated hosts, suggesting that these small tumors may regress. Thus, had we 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 (Campbell et al., 1988), 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. Keratin was observed predominantly in polygonal cells, while vimentin expression is less prominent and is associated with

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 CFP. 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), while predominantly polygonal cells remain after 5 min of trypsinization, (designated CD-5R). Both cell types exhibited p53 immunoreactivity in culture. The CD-3T subpopulation was primarily vimentin-positive and keratin-negative, and had large nuclei. The CD-5R subpopulation contained keratin immunoreactive cells, with little expression of vimentin, and had small nuclei.

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 (Figure 5). However, the CD-5R subpopulation produced fewer tumors (4/12) than the parent in irradiated CFP 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. In contrast, the CD-3T subpopulation was more efficient (12/12) in generating tumors than the parent population (8/12) 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 CFP and irradiated CFP. 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 CFP of hemi-body irradiated mice.

Radiation might contribute to COMMA-D neoplastic progression by causing aberrant immune or endocrine function. Partial (left vs right) body Co^{60}-γ irradiation of anesthetized mice was used to test whether systemic factors from the irradiated host contributed to tumor progression in irradiated CFP. No tumors were found in non-irradiated CFP (n=8), while 3 tumors were observed in irradiated CFP ($P=0.053$). To determine whether the low incidence of tumor formation was due to the systemic effects of anesthesia necessary for hemi-body irradiation, we asked whether anesthesia compromised tumor formation. Anesthetized, whole body irradiated mice formed tumors in 2 of 8 transplanted CFP while un-anesthetized whole body irradiated mice in this experiment formed 7 tumors formed in 8 CFP. Thus, although the low incidence of tumors in the partial body protocol compared to whole body exposures appears to be due to the use of general anesthesia, tumor incidence was restricted to CFP on the irradiated side, indicating that local tissue effects were dominant over the systemic consequences of irradiation.

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.

Aim 3: Define the radiation-induced microenvironment as a function of mammary gland developmental status.

The goal of this study is to determine whether radiation-induced changes in 3, 5, and 8 week old mammary gland undergoing or completing puberty are different from those observed in the adult animals. Dr. Rhonda Henshall joined the lab in April to undertake the studies for this objective. She has completed tissue collection from mice irradiated as a function of age (3, 5 and 8 weeks old) for wholemount and histological analysis. Although there was no evidence of radiation effects on hormonal status in previous studies of adult mice (12 w.o.), it is possible that establishment of ovarian function of young mice might be more susceptible to irradiation. Morphological markers of estrogen effects: cytosmears of the vaginal epithelium and uterine status, were compared to

age-matched controls. No difference was found between the sham-irradiated groups (1 hr and 1 day) and those irradiated 1 hr, or 1, 3, and 7 days before sacrifice. Wholemounts were analyzed as a function of time (1 hr, 1 d, 3 d and 7 d; n=6/time point) following whole body irradiation with 4 Gy for gross morphological endpoints such as endbud number and size, percent of fat pad filling, and width of ducts. Again, no consistent differences were observed between sham-irradiated animals and irradiated animals in any of the gross morphological features. Taken together, these data suggest that gross ovarian function is intake. Studies are beginning to evaluate the character and composition of the ECM of the irradiated mouse mammary gland as a function of animal age and in the different zones of the ductal tree, i.e. endbuds versus adjacent subtending ductal epithelium versus distal epithelium.

Also, as reported last year, we evaluated the physiological mechanisms of TGF-β activation and the consequences of its activity during mammary gland development. Our manuscript describing studies of the physiological regulation of TGF-β activation in normal murine mammary gland reports the novel observation that activation is highly restricted and is differentially regulated by differentiation and estrus. Expansion of these studies to determine the fate and features of TGF-β positive epithelial cells were funded in 1999 by the California Breast Cancer Research Program. The postulated role of TGF-β as a key regulator of normal mammary proliferation will be examined using specific hormonal manipulations and transgenic mouse models.

KEY RESEARCH ACCOMPLISHMENTS

This funding has supported experiments leading to two important and novel observations:

1. *TGF-β activity is spatially restricted and hormonally regulated.*
2. *The abnormal stroma created by radiation exposure promotes the neoplastic potential of COMMA-D mammary cells.*

REPORTABLE OUTCOMES

These data have been reported in part in platform presentations at the following international meetings:

"How Do Tissues Respond to Damage at the Cellular Level?" NASA Investigators Meeting, Brookhaven, NY, June 13, 1999.

"The Role of the Irradiated Microenvironments in Mammary Epithelial Neoplastic Progression" Gordon Conference on Mammary Gland Biology, New London, NH, June 9, 1999.

"Role of Cytokines in Normal Tissue Damage", Biomed Concerted Action on Predictive Assays, Canne, France, October 11, 1998.

"Regulation of Transforming Growth Factor-β Production and Activation During Mammary Gland Development", Epithelial Cell Biology '98, Oxford, England, September 14, 1998.

And abstracts were presented in poster format at:

Barcellos-Hoff, M.H. and S.A. Ravani, Irradiated Stroma is Conducive to Neoplastic Progression of p53 Mutant Mammary Epithelial Cells. International Congress in Radiation Research, Dublin, July, 1999.

Barcellos-Hoff, M.H. The Irradiated Mammary Stroma is Conducive to Neoplastic Progression of Mammary Epithelial Cells Harboring Defective p53. Radiation Research Society Meeting, Louisville, KY; May, 1998.

Manuscripts:
Activation of Latent Transforming Growth Factor-β1 is Regulated During Mammary Gland Development, M.H. Barcellos-Hoff, Lalage Wakefield, G. Shyamala. Submitted.

Irradiated Mammary Gland Stroma Induces Unirradiated Epithelial Cells To Express Tumorigenic Potential, M.H. Barcellos-Hoff and S.A. Ravani. Submitted.

New funding obtained:
Hormonal Regulation of TGF-β During Mammary Development, Principal Investigator, CA-BCRP $451,011 direct, 1999-2001. We propose to use confocal and digital microscopy to correlate spatial and temporal patterns of TGF-β activation with hormone receptors, DNA synthesis, apoptosis and differentiation. We will use transgenic mice to misregulate this expression in specific ways so that complex tissue interactions and morphogenesis can be followed in vivo. This provides the opportunity to examine cause-and-effect relationships between TGF-β and hormone responses in mammary gland. In this proposal we seek to identify the characteristics of both TGF-β negative and positive populations and determine their fate. We will then functionally test two hypotheses: First, that TGF-β is regulated by progesterone will be evaluated using progesterone receptor knockout animals. Second, that TGF-β activity mediates mammary hormone response will be assessed using targeted depletion of TGF-β.

CONCLUSIONS

Transforming growth factor-β1 (TGF-β) inhibits proliferation of mouse and human mammary epithelial cells maintained in cell culture, but as yet direct evidence for this inhibition by endogenous TGF-β has been lacking. Furthermore, since biological activity of TGF-β is controlled by its secretion as a latent complex formed by non-covalent association with latency associated peptide (LAP), the contribution of spatial and temporal constraints on its extracellular activation have not been defined. To localize when and where activation occurs *in situ,* we used dual immunofluorescence detection of anti-LAP relative to anti-chNTGF-β to identify latent versus active TGF-β respectively and digital image analysis to measure their relative expression. Certain epithelial cells exhibited low LAP and high chNTGF-β intensity, which is indicative of latent TGF-β activation. These cells were dispersed throughout the epithelium at certain developmental stages: in endbuds during puberty, at estrus in adult nulliparous mice, and in early pregnancy. We now show that TGF-β haploid genotype leads to accelerated mammary development: duct elongation was accelerated in heterozygotes versus wildtype mice and proliferating cell compartment was increased up to 4 fold in the endbud. Although grossly normal, mammary epithelium from adult, nulliparous animals also exhibited increased proliferation. Taken together, these data provide evidence that endogenous TGF-β is a growth inhibitor during mammary gland development, and that latent TGF-β activation, and thus TGF-β biological activity, is highly regulated *in situ.*

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 (Barcellos-Hoff, 1996; Barcellos-Hoff et al., 1994). We have argued that TGF-β's contradictory role in cancer is because conversion from TGF-β sensitive to TGF-β resistant during tumor progression is a critical juncture in establishing malignant behavior of certain epithelia, like breast (Reiss and Barcellos-Hoff, 1997). TGF-β has been identified as a cancer-promoting agent in wounds (Furstenberger et al., 1989; Sieweke and Bissell, 1994; Sieweke et al., 1990) and recent studies have implicated cyclosporin-induced TGF-β in the increased frequency of neoplasms

following immunosuppressive therapy (Hojo et al., 1999). TGF-β also mediates phenotypic conversion of epithelial cells to vimentin-positive spindle cells (Miettinen et al., 1994). Such a transition is observed in a transgenic keratinocyte model overexpressing TGF-β activity in which the frequency of benign papillomas is suppressed following chemical carcinogenesis, but progression to more malignant spindle cell carcinomas is stimulated (Cui et al., 1996). In the SCp2 cell line, a subclone of late passage COMMA-D (Desprez et al., 1995), TGF-β treatment stimulates the transition from non-tumorigenic keratin-positive cells to tumorigenic, vimentin-positive cells (Galosy, Barcellos-Hoff, Werb & Bissell, unpublished observations). Studies to determine whether the carcinogenesis promoting effect of radiation on stroma is due to TGF-β activation are underway.

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 hematopoeitic malignancies, which have been proposed to result from misregulation of adhesive properties by diseased or genetically aberrant stroma (Gordon et al., 1987). Conversely, the therapeutic benefit of α-interferon in chronic myeloid leukemia has recently been shown to be due in part to the re-establishment of cell-adhesion signals (Bhatia et al., 1996). Greenberger and colleagues proposed a model of indirect γ-irradiation leukemogenesis based on co-cultures of heavily irradiated bone marrow stromal cell lines that selectively bound M-CSF receptor positive unirradiated hematopoietic progenitor cells resulting in selection of tumorigenic subclones (reviewed in (Greenberger et al., 1996)). 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 (Leith and Michelson, 1990). Such studies support the conclusion that radiation has general and persistent effects on stromal function that influences neoplastic progression.

Based on 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 (Barcellos-Hoff, 1998). Studies in cell culture indicate that the frequency of morphological transformation can be modulated by restrictive conditions that select for preexisting cell variants (Chow and Rubin, 1999) and that, conversely, normal cells may actively restrain the expression of the transformed cell phenotype (Bauer, 1996). In vivo studies by Zarbl and colleagues show that mammary tumors with *Hras*1 gene mutations from *N*-nitroso-*N*-methylurea treated rats arose from cells with preexisting *Hras*1 mutations that occurred during early development (Cha et al., 1994). 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 since 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 (Barcellos-Hoff, 1998). Carcinogen-induced microenvironments are not necessarily mutagenic or mitogenic *per se*. Rather, changes in the microenvironment may be conducive to neoplastic progression by disrupting normal cell inhibition of malignant behavior that is regulated through cell-cell contact, cell-ECM interactions and growth factor production. Thus, if ionizing radiation induces a microenvironment that modifies restrictive interactions, then progression may result just as it would if there 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.

These studies provide evidence that supports our hypothesis that the microenvironment elicited by carcinogen exposure are unique, and further that they may act to promote neoplastic progression. As a result, the possibility that microenvironments may be a future target for therapeutic intervention or cancer prevention gains credence.

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