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RADIATION CARCINOGENESIS IN CONTEXT: HOW DO IRRADIATED TISSUES BECOME TUMORS?

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Abstract

It is clear from experimental studies that genotype is an important determinant of cancer susceptibility in general, and for radiation carcinogenesis specifically. It has become increasingly clear that genotype influences not only the ability to cope with DNA damage but also influences the cooperation of other tissues, like the vasculature and immune system, necessary for the establishment of cancer. Our experimental data and that of others suggest that the carcinogenic action of ionizing radiation (IR) can also be considered a two-compartment problem: while IR can alter genomic sequence as a result of DNA damage, it can also induce signals that alter multicellular interactions and phenotypes that underpin carcinogenesis. Rather than being accessory or secondary to genetic damage, we propose that such non-targeted radiation effects create the critical context that promotes cancer development. This review focuses on experimental studies that clearly define molecular mechanisms by which cell interactions contribute to cancer in different organs, and addresses how non-targeted radiation effects may similarly act though the microenvironment. The definition of non-targeted radiation effects and their dose dependence could modify the current paradigms for radiation risk assessment since radiation non-targeted effects, unlike DNA damage, are amenable to intervention. The implications of this perspective in terms of reducing cancer risk after exposure are discussed.

Keywords

Ionizing radiation; carcinogenesis; stromal-epithelial interactions; mammary gland

INTRODUCTION

A fundamental challenge in radiation research related to human health is to predict the biological impact of exposure to low dose (<0.1 Gy) ionizing radiation (IR). Excess cancers have been observed in the Japanese atomic-bomb survivors at doses of 0.1 to 4 Gy, which are 40 to 1600 times the average yearly background levels in the United States. The excess risks vary significantly with gender, attained age, and age at exposure for all solid cancers as a group and many individual sites as a consequence of the atomic bomb (Preston et al. 2007). It has been estimated that if radiation exposure occurs at age 30, the solid cancer rates at age 70 is increased by about 35% per Gy (90% CI 28%; 43%) for men and 58% per Gy (90% CI 43%; 69%) for women (Preston et al. 2007). Predicting cancer risk in populations exposed to doses lower than ~0.1 Gy is limited by statistical considerations. Therefore, radiation risk models

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extrapolate in the region below which epidemiological data are robust using an assumption of linearity. The linear-no-threshold (LNT) regulatory paradigm is based in large part on observations that cancer incidence increases with increasing dose above 0.1 Gy, as well as pragmatic, regulatory and societal considerations to protect the population.

A recent review study of the National Academy of Sciences (BEIR VII) concluded that human health risks continue in a linear fashion at low doses without a threshold so the smallest dose has the potential to increase risk in humans (NAS/NRC 2006). The scientific rationale for linearly extrapolating radiation health effects is underpinned by biophysical theory of how energy interacts with DNA, which is thought to be the major biological target. This area of radiation biology has made significant progress in identifying the critical mechanisms, processes and pathways by which DNA is damaged, repaired or misrepaired. The efficiency and frequency by which IR induces mutations and chromosomal aberrations is thought by most to be the best surrogate of its carcinogenic potential. This is in part because there is a clear mechanistic understanding of these genomic modifications via energy deposition, and because these events are strongly associated with cancer. A fundamental principle of target theory is that the effect (e.g., DNA damage, cell kill, mutation) is linear or linear/linear-quadratic as a function of dose due to biophysical considerations that energy deposition (i.e., dose) is proportional to damage. In terms of immediate damage, so-called targeted radiation effects, this conclusion is very well supported for DNA damage that can be measured directly or indirectly over several logs of radiation exposure (1–100 Gy).

However, biological responses to radiation damage quickly evolve and amplify in a nonlinear manner, particularly at low doses, which has been broadly documented both in cell culture and in vivo (reviewed in (Brooks 2005; Wright and Coates 2006). There are now myriad experimental reports that low dose radiation (1) alters the response of cells and tissues to subsequent challenge dose (i.e., adaptive responses), (2) affects daughter cell fates such as differentiation and senescence, (3) induces long-range signals that affect non-irradiated cells, and (4) generates a state of chronic genomic instability (GIN). Although there are several definitions of nontargeted effects, we define nontargeted effects as those that are inconsistent with either direct energy deposition, such as bystander phenomenon (Kaplan et al. 1956b; Hei et al. 1997; Barcellos-Hoff and Ravani 2000; Mothersill et al. 2001), or those that are exhibited in the daughters of irradiated cells, but not mediated by a mutational mechanism, such as radiation-induced GIN (Kadhim et al. 1992;Kadhim et al. 1994; Kadhim et al. 1995; Clutton et al. 1996; Limoli et al. 1997) and persistent phenotypic changes (Herskind and Rodemann 2000; Rave-Frank et al. 2001; Park et al. 2003; Tsai et al. 2005). Although the extent to which these phenomena reflect different molecular mechanisms is not clear, experimental results to date suggest that significant deviation from linearity at low doses may impact the ability to predict cancer risk in humans (Baverstock 2000; Wright 2000; Barcellos-Hoff and Brooks 2001; Huang et al. 2003; Little 2003).

Do nontargeted radiation effects alter the predicted dose dependence of radiation carcinogenesis at low doses? Considerable debate has arisen regarding the relevance of nontargeted effects in radiation protection paradigms. Indeed the French Academy of Medicine concluded that the evidence is compelling showing the mechanisms of response to low dose/dose rate are significantly different from those operating at high doses (2005). They propose that the current policy may lead to an overestimation of risks. Biologically relevant, nonlinear radiation responses could have significant implications for the LNT regulatory assumption, but it is unlikely that they will be incorporated into the regulatory perspective unless a more comprehensive biological paradigm of radiation carcinogenesis is generally accepted.

Our overarching hypothesis is that cancer emerges as a result of a complex, but ultimately predictable, interplay between targeted and non-targeted effects in the context of host genetics

and physiology (Barcellos-Hoff 2007). Just as DNA damage elicits a dramatic transition in signaling within a cell, each irradiated tissue has its own set of signals and cell types, distinct from those of unirradiated tissue and different from other irradiated tissues. The sum of these events, occurring in different organs and highly modulated by genotype, predicates the consequence to the organism. We propose that radiation exposure culminates in cancer as a result of oncogenic mutations from targeted DNA damage that occur in the context of the biology of irradiated tissues driven in large part by nontargeted radiation effects (Barcellos-Hoff 2005;2007). The dose dependence of the former is well-established; the dose dependence of the latter is crucial to understanding the risk for radiation associated carcinogenesis.

CARCINOGENESIS IN CONTEXT

Despite the prevalence of overt cancer in humans (one of three Americans will be diagnosed during their lives), cancer is much more frequent at the tissue level according to autopsy studies. At age 50, 1 of 4,000 people will be diagnosed with thyroid cancer although 99% of autopsy specimens contain frank malignancies (Tulinius 1991). Similarly, many more Western men compared to Japanese men develop clinical prostate cancer by age 60, even though carcinomas are equally prevalent in autopsy specimens (Stemmermann et al. 1992). Autopsy data also show that breast cancer is much more prevalent at the tissue level than is clinically evident (Nielsen et al. 1984; Nielsen 1989). Thus, random genetic changes occur sufficiently frequently to produce malignant cells in large part as a result of normal living, but do not progress at the tissue level. It is thought that these nascent cancers fail to recruit normal cells into the neoplastic process [reviewed by (Folkman et al. 2000)].

There is growing recognition that as a disease, cancer results from a systemic failure in which many cells other than those with oncogenic genomes determine the frequency of clinical cancer. Pioneering studies by Mintz and Pierce during the 70s showed that malignancy could be suppressed by normal tissues (Mintz and Illmensee 1975; Pierce et al. 1978). Dvorak proposed that cancer is analogous to a wound that never heals (Dvorak 1986), an idea that implicates the importance of tissue remodeling and inflammation, both of which involve the functions of tissues. It has become increasingly evident that tissue structure, function and dysfunction are highly intertwined with the microenvironment during the development of cancer (reviewed in) and that tissue biology and host physiology are subverted to drive malignant progression (Coussens and Werb 2001; Bissell et al. 2002; Barcellos-Hoff and Medina 2005). Recent examples that have identified specific signals and cells that contribute to carcinogenesis are discussed in the following section. These experimental models provide strong mechanistic support for dominant control by the microenvironment even in highly efficient carcinogenesis driven by strong oncogenic programs.

Coussens and colleagues employed a transgenic mouse model that expressed the human papillomavirus type 16 (HPV16) early region genes under the control of the keratin 14 promoter in order to examine the link between chronic inflammation and skin cancer (de Visser et al. 2006). They hypothesized that interactions between adaptive immune cells and initiated, “at risk,” cells were determinants of skin cancer progression. This was tested by crossing the transgenic model with a RAG-1−/− mouse that lacks mature B and T lymphocytes. Unlike the K14-HPV16 mice that exhibit leukocyte recruitment and chronic inflammation in premalignant skin, HPV16/RAG-1−/− mice did not possess these features or the subsequent parameters necessary for full malignant progression (i.e., release of proangiogenic factors, activated vasculature, and hyperproliferation of oncogene-positive keratinocytes). Transfer of either B lymphocytes or serum from K14-HPV16 mice effectively restored the chronic inflammation and malignant progression in HPV16/RAG-1−/− mice. Interestingly, B lymphocytes did not infiltrate the skin tumors in this study, but were found to exert their effects by depositing immunoglobulins in a paracrine fashion.

A study by Pollard and colleagues used a mammary restricted polyoma middle T oncoprotein, of which tumors undergo pre-malignant stages prior to advanced carcinomas (Lin et al. 2001). The investigators examined the kinetics and contribution of tumor associated macrophages in the development of the vasculature that is essential for progression, otherwise known as the "angiogenic switch." Enhanced macrophage infiltration was found to always precede the increase in vessel density that characterized the transition between pre-malignant and early carcinoma stages. Genetic depletion of macrophages by homozygous deletion of the macrophage growth factor, CSF-1, resulted in a delay in both angiogenic switch and malignant progression, suggesting that macrophages regulated angiogenesis. In addition, transgenic over expression of CSF-1 under the mammary specific mouse mammary tumor virus promoter in this model resulted in very early recruitment of macrophages. Importantly, accompanying this enrichment was the development of a late-stage vessel density during the early pre-malignant stage of hyperplasia. Thus, premature macrophage recruitment was sufficient to stimulate a degree of angiogenesis that could support a late-stage carcinoma, indicating that angiogenic activity is not simply in response to enhanced tumor size (and hypoxia) but was controlled by the host, independent of tumor stage.

Evan and colleagues engineered a OH-Tamoxifen-inducible form of the transcription factor c-Myc, restricted to islets of the mouse pancreas by the proximal insulin promoter, as a model to study the in vivo mechanisms of its oncogenic potential (Shchors et al. 2006). They found that sustained c-Myc activation drives proliferation of β-cells of the islets and also indirectly increases proliferation of endothelial cells. The cytokine IL-1β, which was transcriptionally induced after activation of c-Myc, was determined to be necessary and sufficient for the angiogenic effects of c-Myc activation. Systemic administration of neutralizing antibodies against IL-1β had no effect on Myc-induced β-cell proliferation, but severely impaired the activation and redistribution of the angiogenic factor VEGF-A, which remains dormant in the islet extracellular matrix until activated. Thus, though c-Myc exerts a potent proliferative push in β islet cells, an important aspect of its action in tumor promotion is the production of IL-1β, which serves as a paracrine trigger to modify the microenvironment around the islet.

Human epithelial cells are also subject to the influence of the microenvironment. A human mammary model developed by Weinberg underscores both (1) the requirement for the appropriate microenvironment in the ability of epithelial cells to perform in a tissue-appropriate manner and (2) a critical role of abnormal stroma in cancer promotion (Kuperwasser et al. 2004). The model employs the mouse mammary gland as the host for human fibroblasts, which, when irradiated in vitro, take up permanent residence in the cleared fat pad. This humanized stroma supports the growth and morphogenesis of subsequently transplanted human mammary epithelial organoids. Proper ductal morphogenesis depends on the admixture of primary normal breast fibroblasts to these organoids prior to engraftment into humanized fat pads. Although specimens from most individuals gave rise to apparently normal ductal outgrowths, one specimen gave rise to hyperplastic growth, suggesting the presence of neoplastically initiated, but dormant, cells. When that preparation was transplanted in a murine stroma humanized with stromal cells engineered to over express either HGF or TGFβ1, the organoids developed into growths closely resembling human comedo-type and basal-type invasive carcinomas, respectively. The authors conclude that an altered stromal environment can promote human breast cancer formation through abnormal epithelial cells present, but dormant, in the normal human breast.

These examples provide specific mechanisms at play in carcinogenesis driven by experimentally induced oncogenes. Radiation carcinogenesis is much more challenging to similarly dissect given the random nature of initiation, the genetic variation between individuals, and the susceptibility of a particular tissue. We propose that cancer initiation (defined as mutations resulting from unrepaired or misrepaired DNA damage caused by IR) is

only half the story, and that radiation-induced host biology is a critical action of radiation as a carcinogen and in the development of clinical cancer. Unlike the random interaction of energy with DNA, resulting in damage and mutation, tissue response to radiation is orchestrated, predictable and may ultimately be amenable to intervention.

RADIATION CARCINOGENESIS

Although the prevailing risk paradigm focuses on radiation-induced DNA damage leading to mutations in susceptible cells, numerous studies over the last 50 y have provided evidence that radiation carcinogenesis is more complex. Terzaghi-Howe demonstrated that the expression of dysplasia in vivo and neoplastic transformation in cultures of irradiated tracheal epithelial cells is inversely correlated with the number of cells seeded (Terzaghi and Little 1976; Terzaghi and Nettesheim 1979; Terzaghi-Howe 1986; Terzaghi-Howe 1989) and identified TGFβ as a key mediator (Terzaghi-Howe 1990). Greenberger proposed in 1996 that irradiated stromal cells function as biologic tumor promoters in leukemia through their release of reactive oxygen species, and production of altered adhesion molecules or growth factors that block apoptosis and induce DNA strand breaks in closely associated self-renewing stem cells (Greenberger et al. 1996c). Long-term bone marrow cultures were used in which irradiated bone marrow stroma actively contributes to leukemogenesis via growth factors, reactive oxygen and altered adhesion molecules that regulate the expansion of hematopoietic stem cells. The bone marrow stromal cell alterations of CBA/B mice irradiated with 200 cGy persisted 6 mo after explant of the cells to culture (Greenberger et al. 1996a). Irradiated bone marrow stromal cell line D2XRII expresses persistently altered fibronectin splicing, increased expression of several transcriptional splice variants of macrophage-colony-stimulating factor, and increased TGFβ (Greenberger et al. 1996b).

Extensive studies were published by Kaplan and colleagues in a series of four papers in the 50s. C57BL mice are very susceptible to thymic lymphomas after radiation exposure. Young mice underwent thymectomy, and 2–7 d later received the first of four consecutive doses of 168 cGy, spaced apart by 8-d intervals. Several hours after the last irradiation, a single thymus from a non-irradiated mouse was transplanted subcutaneously under the right chest or upper abdomen of each of the previously thymectomized, irradiated hosts. Tumors were then tracked by palpation for 15 mo thereafter. Amazingly, the incidence and latency of the thymic lymphomas arising from the grafts matched that observed in irradiated, intact mice (39% and 214 d, respectively). Furthermore, the tumors were histologically identical to those found in the intact mice, and exhibited a similar pattern of metastasis (Kaplan et al. 1956b). This study showed that radiation-induced thymic lymphomas can occur even when the grafted thymus was never exposed to radiation, suggesting a systemic effect of tumor induction inherent to the host.

This systemic mechanism of tumor induction was elucidated in their second study, which showed that shielding a thigh of the host during irradiation or promptly injecting fresh bone marrow into the host shortly after the last irradiation could neutralize the tumor-inducing effect of IR. Using the same experimental approach as in the first study, but varying the time of implantation after the last irradiation, the authors showed that the tumor promoting effect of IR through the host persisted for up to 8 days, yielding tumor incidences that were not significantly different from implantations performed 1–3 hrs post-irradiation (Kaplan et al. 1956a).

In the third study, Kaplan and colleagues examined the physiological status of the unirradiated thymic graft after it was transplanted into a previously thymectomized and irradiated host. Massive necrosis was observed at 24 h after implantation, with only a few surviving cells under the capsular membrane. These regions of survival, however, would eventually be repopulated

within the course of the next 14 d into a graft with a regenerated cortex. At this time point grafts increased in total size and even reformed lobes, though not always two nor complete lobes. Comparing graft regeneration in thymectomized, irradiated or unirradiated hosts revealed that prior radiation exposure impaired regeneration. Consistent with the finding that bone marrow injection neutralized tumor induction through the irradiated host, thigh-shielded mice exhibited an identical degree of graft regeneration as observed in unirradiated mice, while unshielded mice had significantly impaired regeneration (Carnes et al. 1956). The authors thus concluded that a systemic bone marrow factor in the host was necessary for proper regeneration of unirradiated thymic grafts, and that radiation compromised this factor in the host as a mechanism of tumor induction.

In a fourth study, Kaplan and colleagues provided conclusive evidence that the tumors that arose in the unirradiated thymic grafts were indeed composed of donor cells and not invading host cells that had received radiation. The susceptible C57BL strain of mice was crossed with the C3H strain, which is resistant to radiation-induced lymphomas, to generate an F1 hybrid. Using the same experimental approach of transplantation into previously irradiated hosts, the authors revealed that though host irradiation could induce lymphomas, the genetic background of the graft donor heavily determined tumor incidence. Hosts bearing grafts from the susceptible C57BL or F1 hybrid strains had more tumors than those bearing grafts from C3H donors, thus indicating that susceptibility to radiation-induced lymphomas was a property that was inherent to the thymus, even though the mechanism of induction can occur through the host. Lastly, to prove that tumors induced through host exposure, but arising in the graft were truly cells from the unirradiated implant, tumor fragments were excised from grafts that were either C57BL or F1 hybrid, and then implanted, subcutaneously or intraperitoneally, back into hosts from each of the three genetic backgrounds. The tumor fragments from C57BL grafts only grew in the C57BL and F1 host, not in the C3H host; and fragments from hybrid grafts grew only in hybrid hosts. Thus, the rejection of tumor fragments when they were placed into hosts of a different background shows that the tumor cells were derived from the graft and not the host (Kaplan et al. 1956c). This series of papers highlight the host as an effective target of radiation in the induction of thymic lymphomas in grafts that were never irradiated. Similarly, a study of skin carcinogenesis done by Billingham and colleagues used the carcinogen methylcholanthrene to determine which compartment was the site of carcinogenic action in mouse skin. Skin grafts of various thicknesses (including or excluding hair follicles) from carcinogen-treated sites were transplanted to untreated sites in the same animal. Such an approach revealed that the underlying dermis layer conferred equivalent tumorigenic potential, even if the overlying epidermis was untreated. Tumors occurred when untreated grafts were transplanted into treated dermis, but not when treated grafts were placed into untreated dermis (Billingham et al. 1951).

Ethier and Ullrich showed that dissociation of cells from mouse mammary glands irradiated with 1 Gy and transplanted 24 h after exposure to unirradiated mice increased the frequency and persistence of dysplasia over that of intact tissues (Ethier and Cundiff 1987; Ethier et al. 1987), suggesting that normal tissue interactions suppress neoplastic potential. Clifton and colleagues showed that 1/100 clonogens (i.e., those capable of growing in vivo) dissociated from irradiated rat mammary glands undergo initiation when transplanted into unirradiated tissue, a frequency that is inconsistent with radiation-induced mutation.

IR-induced rapid remodeling of the mammary microenvironment led us to hypothesize that the irradiated stroma modified tumorigenic potential (Barcellos-Hoff 1993, Barcellos-Hoff 1998, Barcellos-Hoff 1998). To test this hypothesis, we created a radiation chimera by transplanting unirradiated, preneoplastic mammary cells to the mammary glands of irradiated hosts (Barcellos-Hoff and Ravani 2000). The undeveloped mammary epithelium is surgically removed at puberty, the animal irradiated, and some time later non-irradiated mammary

epithelial cells are transplanted into the irradiated host. These studies used COMMA-1D mammary epithelial cells, which undergo mammary morphogenesis when transplanted into a 3-wk old mammary gland. They are non-tumorigenic if injected into the cleared fat pads of 3 wk old mice, subcutaneously in immature and adult mice, or into nude mice. Although clonal in origin, COMMA-1D cells harbor two mutant Trp53 alleles that may confer neoplastic potential (Jerry et al. 1994). When transplanted into mice irradiated 1–14 d earlier with 4 Gy, outgrowths rapidly developed tumors, ranging from a peak of 100% at day 3 and twice that of sham-irradiated mice at 14 d postirradiation. Furthermore, tumors from irradiated animals were nearly five times larger than the few tumors that arose in sham-irradiated hosts, indicating that tumor biology, as well as frequency, was affected. These data support the idea that high dose radiation promotes carcinogenesis by inducing a hospitable tissue environment.

If the host microenvironment created by radiation can promote neoplastic progression in unirradiated epithelial cells, then events “outside of the box” do significantly increase cancer risk. We believe that this adverse “bystander effect” of irradiated cells on unirradiated cells is due to extracellular signaling from the microenvironment that supports progression. The effect of the irradiated microenvironment on neoplastic progression persisted for several weeks and appears to be independent of systemic radiation effects (as tested by hemi-body irradiation), which support the hypothesis that non-mutagenic effects of radiation can contribute significantly to radiation carcinogenesis in vivo. If key signals that promote carcinogenesis in irradiated tissues are identified, then the irradiated microenvironment can be a therapeutic target to mitigate the long-term consequences of inadvertent radiation exposures.

CONTRIBUTION OF TGFβ TO CARCINOGENESIS

Radiation-induced DNA damage elicits checkpoints for genome integrity that coordinate with the cell cycle machinery to ensure accurate transmission of genetic information. These checkpoints are complemented by preemptive apoptotic triggers that eliminate damaged cells in order to maintain tissue integrity. Such cellular responses to damage must be integrated within the context of multicellular tissues to maintain homeostasis. Radiation also rapidly induces extracellular signaling via growth factors and cytokines that regulate stromal remodeling, vascular integrity and inflammatory responses (reviewed in Hallahan et al. 1993; McBride 1995; Barcellos-Hoff 1998; Dent et al. 2003). In particular, IR induces the activation of TGFβ, a growth factor that is produced and widely distributed extracellularly as a latent complex (Barcellos-Hoff et al. 1994; Ehrhart et al. 1997). TGFβ mediates epithelial fate decisions by regulating proliferation and apoptosis (reviewed in Derynck et al. 2001).

TGFβ has been widely implicated in radiation responses. Terzaghi-Howe showed that TGFβ produced by the differentiated normal epithelial cells inhibited the growth and phenotype of radiation-transformed cells (Terzaghi-Howe 1986). Bauer described three distinct, but competing, roles for TGFβ during transformation (reviewed in Haufel et al. 1999): TGFβ actually helps maintain the transformed state of mesenchymal cells, but it also enables nontransformed neighbors to recognize transformed cells and trigger an apoptosis-inducing signal. Bauer and colleagues recently showed that the latter two processes are enhanced following very low radiation doses (Portess et al. 2007).

Similarly, we postulated a positive net role of the extracellular TGFβ activity induced by radiation in vivo and in vitro (Barcellos-Hoff and Brooks 2001). We used mice and primary cultures to determine the effects of TGF∃ on radiation-induced molecular events and cell fate decisions (Ewan et al. 2002, Kirshner et al. 2006). Radiation-induced apoptosis is significantly reduced in *Tgfβ1* heterozygote embryonic liver, skin, and adult mammary gland while *Tgfβ1* null embryos fail to undergo either apoptosis or inhibition of the cell cycle in response to 5 Gy (Ewan et al. 2002).

The prototype DNA damage response is the one mobilized by the highly cytotoxic double-strand break (DSB) induced by IR (Bassing and Alt 2004). The molecular response to this damage results in the activation of cell cycle checkpoints, which temporarily halt the cell cycle until the damage is repaired (Lukas et al. 2004). The mechanism that allows this rapid dissemination of the damage alarm is based on a signal transduction pathway that begins with sensor/activator proteins that sense the damage or possibly the chromatin alterations that follow damage induction. These proteins play a major role in the activation of the transducers, which further convey the signal to multiple downstream effectors (Bakkenist and Kastan 2004). The primary transducer of the DSB alarm is the nuclear protein kinase ataxia telangiectasia mutated (ATM) checkpoint kinase (Shiloh 2003, Kurz and Lees-Miller 2004). ATM is missing or inactivated in patients with ataxia-telangiectasia (A-T), which is complex and characterized by extreme sensitivity to ionizing radiation and DSB-inducing agents. In response to DSBs, ATM is activated and phosphorylates numerous substrates, thereby modulating the processes in which these proteins are involved. ATM targets specifically serine or threonine residues followed by glutamine (the “SQ/TQ” motif) (Bakkenist and Kastan 2003; Shiloh 2003; Kurz and Lees-Miller 2004). ATM activation is mediated and/or reflected by auto-phosphorylation at serine 1981 (1987 in mice), and a fraction of activated ATM binds to the DNA damage sites (Andegeko et al. 2001; Bakkenist and Kastan 2003).

ATM precisely controls its downstream pathways, often by influencing the same process from several different directions (e.g., the cell-cycle checkpoints), each of which is governed by several ATM-mediated pathways (Shiloh 2003). Notably, in addition to ATM’s versatility as a protein kinase with numerous substrates, the ATM web contains protein kinases that are themselves capable of targeting several downstream effectors simultaneously, and as such concomitantly control subsets of pathways (e.g., the Chk1 and Chk2 kinases). A prototype example is the ATM-mediated phosphorylation and subsequent stabilization of the p53 protein, a major player in the G1/S cell cycle checkpoint on one hand and in damage-induced apoptosis on the other (Meek 2004).

Recent studies demonstrate that TGFβ is an essential regulator of the intrinsic ATM response to DNA damage in epithelial cells (Kirshner et al. 2006). Either chronic TGFβ depletion by gene knockout or transient depletion by TGFβ neutralizing antibody reduced phosphorylation of p53 serine 18 in the irradiated mammary gland (Ewan et al. 2002). Together, these data implicate TGFβ in the genotoxic stress program of epithelial tissues. We then established that treatment with TGFƎ restored the molecular and cell fate response and that we could phenocopy the genetic model in human cells using a small molecule inhibitor of the TGFƎ type I receptor. Irradiated primary epithelial cultures from *Tgfβ1* null murine epithelial cells or nonmalignant human mammary epithelial cell lines in which TGFβ ligand or signaling was blocked exhibited 70% reduction of ATM kinase activation, failed to auto-phosphorylate, and neither growth arrested or underwent apoptosis in response to radiation (Kirshner et al. 2006). TGFβ treatment prior to radiation restored damage responses, supporting a specific requirement for TGFβ signaling in the genotoxic stress programs via modulation of ATM kinase activation.

Rather than being independent, the intracellular and extracellular damage response programs are functionally linked in epithelial cells. Inability of the cell to properly repair DNA damage caused by radiation or other DNA damaging agents can lead to genomic instability and increased cancer frequency and progression (reviewed in Khanna and Jackson 2001; Kastan and Bartek 2004). Likewise, epithelial cells deficient for TGFβ show genomic instability (Glick et al. 1996), increased tumor progression (Glick et al. 1993), and are haploid insufficient for carcinogenesis (Tang et al. 1998).

Radiation-induced genomic instability that occurs in clonally expanded, finite life span, normal human mammary epithelial cells (HMEC) as measured by aberrant karyotypes and supernumery centrosomes (Sudo et al. 2008). As expected, based on its role in DNA damage response, TGFβ inhibition increased genomic instability in irradiated and control HMEC (Maxwell et al. 2008). However, TGFβ treatment to genomically unstable HMEC actually reduced GIN after the fact, as was originally shown by Glick using PALA resistance in primary keratinocytes (Glick et al. 1996). Our studies in HMEC revealed that TGFβ selectively deleted genomically unstable cells via p53-dependent apoptosis, resulting in an overall increase in population stability. Thus, endogenous TGFβ suppresses radiation-induced and spontaneous genomic instability, but attenuation of TGFβ signaling permits survival of genomically unstable cells. Thus, experimental models in which TGFβ activity is limited (e.g., clonal culture) may more readily demonstrate GIN because this extracellular surveillance mechanism is inefficient. The interaction between intrinsic radiation damage response and the extrinsic control via microenvironment determine the prevalence of unstable human cells (Maxwell et al. 2008) and transformed rodent cells (Terzaghi-Howe 1989; Portess et al. 2007).

However, there is more to the story. The progeny of irradiated HMEC embedded in reconstituted basement membrane undergo disrupted alveolar morphogenesis if exposed to TGFβ (Park et al. 2003). Single irradiated HMEC gave rise to colonies exhibiting decreased localization of E-cadherin, β-catenin, and connexin-43, which are proteins necessary for the establishment of cell polarity and communication. Severely compromised acinar organization was manifested by most irradiated HMEC progeny, arguing against a mutational mechanism. We compared the effect of IR on ability of MCF-10A and HMT3522 S1 cell lines to that of 184 extended life span HMECs, which are completely stable by both karyotype and comparative genomic hybridization. Surprisingly, all three non-tumorigenic HMEC are susceptible and undergo disrupted acinar morphogenesis and loss of E-cadherin. These data point to a heritable, non-mutational mechanism whereby IR compromises cell polarity and multicellular organization. Notably, we found a dose response similar to that observed in nontargeted phenomena, [(i.e., a steep response at low dose (<10 cGy)] followed by a plateau. Is this a novel radiation response exhibited only in culture? Interestingly, urinary bladder carcinogenesis in humans exposed to long-term low-dose radiation exhibit significant increases of TGFβ1 and altered localization of E-cadherin/β-catenin complexes (Romanenko et al. 2006). Also, Arteaga and colleagues showed that IR-induced TGFβ promotes metastatic breast cancer in vivo (Biswas et al. 2007).

The underlying mechanism of TGFβ mediated disrupted morphogenesis by irradiated cells is epithelial to mesenchymal transition (EMT). EMT is the product of the intersection of the intrinsic response to IR, in this case activation of the MAP-K pathway, and chronic TGFβ signaling from the microenvironment (Andarawewa et al. 2007). Although radiation-induced TGFβ was demonstrable by media transfer, endogenous radiation-induced TGFβ was insufficient to drive EMT, which underscores the sources and duration of TGFβ activity in tissues as an important determinant of effect. As found with morphogenesis, and consistent with a non-targeted effect, irradiation with either 2 or 200 cGy appear to be equally effective in priming HMEC to undergo TGFβ mediated EMT (Andarawewa et al. in preparation). Thus, while endogenous TGFβ primarily eliminates radiation-induced genomically unstable cells via apoptosis, exogenous chronic exposure promotes phenotypic instability.

Indeed, TGFβ promotion of carcinogenesis is often ascribed to its ability to drive phenotypic switching (Han et al. 2005; Zavadil and Bottinger 2005). Overexpression of constitutively active TGFβ can induceEMT during tumor progressionin vivo (Portella et al. 1998) and the overexpression of TGFβ has been associated with poor prognosis of many human cancers (Bierie and Moses 2006). In support of a dominant pro-carcinogenic action, polymorphisms that appear to increase TGFβ production are associated with risk of advanced cancer. Compared

with other genotypes, high *TGFβ1* producer genotypes were associated with an increased risk of colorectal adenoma (Berndt et al. 2007), nasopharyngeal cancer (Wei et al. 2007), malignant melanoma (Nikolova et al. 2007) and lung cancer (Kang et al. 2006). While the role of *TGFβ1* polymorphisms in breast cancer is complex, a recent large consortium confirmed increase in breast cancer risk associated with a polymorphism that increases protein production (Cox et al. 2007a).

TGFβ is classically described as a tumor suppressor since it is a profound inhibitor of epithelial cell proliferation. Consistent with this, *Tgfβ1* heterozygote mice, which express only 10–30% of wild type protein levels, in combination with oncogene expression or chemical carcinogen exposure, exhibit increased tumor incidence and size (Tang et al. 1998) as well as decreased tumor latency (Glick et al. 1994; Forrester et al. 2005). TGFβ is implicated in tumor processes that affect angiogenesis (Ueki et al. 1992), reactive stroma (Iozzo and Cohen 1994; Mahara et al. 1994), and immunosuppression (Li et al. 1993; Hojo et al. 1999). Based on the paradigm in which TGFβ acts as a tumor suppressor, one would expect TGFβ compromised mice, like those in which the TGFβ receptor was floxed (Bhowmick et al. 2004), to be extremely cancer prone. However, many labs including ours have observed that spontaneous cancer is not increased in *Tgfβ1* heterozygote mice, even when aged for 2 y (unpublished data). *Tgfβ1* null mice crossed onto an immune deficient background (which prevents neonatal death from gross inflammatory disease shortly after birth (Shull et al. 1992), have little evidence of spontaneous cancer when housed under germ-free conditions. These mice do develop gastrointestinal cancer under standard mouse husbandry but not when housed under germ-free husbandry (Engle et al. 2002), indicating that TGFβ mediates the interactions between inflammation and epithelial cancer. The lack of spontaneous cancer in mice that have reduced TGFβ appears to contradict the thesis that TGFβ acts primarily as a tumor suppressor in the intact organism. Our unpublished data (Nguyen and Barcellos-Hoff) using the radiation chimera model of *Tgfβ1* heterozygote Balb/c mice transplanted with *p53 null* mammary epithelium suggests that host TGFβ is a major mediator of radiogenic cancer. Given *TGFβ1* polymorphisms in humans, and the complex roles TGFβ plays in tissues, it is clear that TGFβ warrants further investigation in the context of radiation exposure.

FUTURE DIRECTIONS IN RADIATION BIOLOGY

Many have argued, even at the height of focus on identifying critical mutations, that disruption of the cell interactions and tissue architecture are primary drivers of carcinogenesis (Rubin 1985; Barcellos-Hoff 1998; Sonnenschein and Soto 2000; Bissell and Radisky 2001, Wiseman and Werb 2002). Recent experiments demonstrating the key role of normal cells in cancer (Bhowmick et al. 2001; Kuperwasser et al. 2004; Maffini et al. 2004, de Visser et al. 2006) offer provocative evidence that microenvironment composition determines whether cancer ensues following mutational activation of oncogenes or loss of tumor suppressors. Since an oncogenic genome can be effectively suppressed by normal tissues, and radiation-induced microenvironments promote oncogenesis, then understanding nontargeted mechanisms can readily lead to testable hypotheses, and possible interventions, for health risks in future populations. Strategies that block the effects of IR mediated by the microenvironment are likely to significantly reduce long term cancer risk.

Nontargeted radiation phenomena are also an impetus to reevaluate whether extrapolation of risk from high to low doses, or from acute to chronic exposures, is reasonable. Our experimental data and that of others suggest that the action of radiation as a carcinogen is a two-compartment problem: IR alters the genome of the target (e.g., epithelium), in the context of radiation-induced phenotypes of other cells of the tissue. Therefore cancer following radiation is the end result of both mutations and altered signaling via the microenvironment. At least three aspects of cancer are underappreciated when DNA damage and mutation is used as the scientific

rationale for LNT extrapolation of radiation risks from high to low doses. First, recognition that IR alters cell phenotype as well as genotype (reviewed in Barcellos-Hoff et al. 2005). Second, that initiated cells progress in the context of accessory/host cells, which ultimately determines whether cancer progresses (Coussens and Werb 2002). And third, that specific signals, like TGFβ, play a global role in orchestrating tissue functions (Akhurst 2002). Even if the nature and dose dependence of these processes are not as yet completely understood, there is more than sufficient evidence that they, in conjunction with DNA damage, determine cancer risk at high doses.

Multicellular responses and extracellular signaling following radiation exposure are integral, rather than secondary in evaluating radiation risks. Some dose responses show increased response with increased dose (e.g., TGFβ activation in situ (Ehrhart et al. 1997)) while others like phenotypic responses appear to act like switches at low dose [e.g., EMT (Park et al. 2003) and unpublished data]. If cancer is a function of both genomic alterations and microenvironment disruption, then it is critical to ascertain whether microenvironmental changes are linearly related to direct energy deposition. Clearly defining the complex processes that lead to cancer is important in order to accurately predict radiation health effects. Although a biological model in which radiation risk is the sum of dynamic and interacting processes may not readily replace a pragmatic risk model, it could provide the impetus to reassess our assumptions about radiation health effects in populations. Furthermore, it can possibly spur new approaches to intervention or countermeasures.

Systems biology attempts to quantitatively evaluate interactions and relationships to predict complex events. Systems *radiation* biology could be an approach to integrate information determined by experimentation across different times and scales. A key property of a system is that some phenomena emerge as a property of the system rather than the parts. Modeling that analyzes the irradiated tissue/organ/organism as a system rather than a collection of noninteracting or minimally interacting cells could provide support for the idea that cancer is an emergent phenomenon of a perturbed system (Barcellos-Hoff 2007). Given the current research goal to understand the consequences of high versus low radiation exposures in humans, broadening the scope of radiation studies to include systems biology concepts should benefit risk modeling of radiation carcinogenesis.

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