Epithelial Cell Cycling Predicts p53 - Repsoniveness to γ-Irradiation During Post-Natal Mammary Gland Development

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INTRODUCTION

The multiphasic post-natal developmental processes occurring in the murine mammary gland are exquisitely orchestrated by endocrine regulation. Circulating steroid hormones estrogen and progesterone, act on the mammary gland to cause rapid ductal elongation and infiltration into the surrounding adipose tissue, ultimately establishing the mature resting gland (Nandi et al., 1958; Delouis et al., 1980; Ceriani et al., 1974; Woodward et al., 1998). Subsequent to maturation, the quiescent gland responds to the hormones of pregnancy, specifically estrogen, progesterone, placental lactogen and prolactin, with an initial burst of epithelial cell proliferation on day 4 of pregnancy, followed by a second proliferative peak at approximately day 12 (Traurig et al., 1967). By this time, circulating levels of the lactogenic hormone prolactin promote transcription of β-casein mRNA, the hallmark of mammary epithelial differentiation, as synthesis of milk proteins begins (Robinson et al., 1995). At parturition, lactation is established and reaches maximum yield by day 5. Within 10 days of weaning, the mammary gland undergoes massive structural remodeling during which over 80% of its differentiated epithelium is deleted by apoptosis (Walker et al., 1989; Strange et al., 1992). These developmental phases in the murine mammary gland mimic closely the stages of human breast development (Cardiff et al., 1999), making it a useful model of both mammary gland development and susceptibility to tumorigenesis (Medina, 1996; Ghebranious and Donehower, 1998).

Numerous carcinogens may initiate cellular transformation (Otteneder and Lutz, 1999; Vineis et al., 1999), and exposure to ionizing radiation is one of the most widely studied (Ullrich, 1991; Barcellos-Hoff, 1998; Ron, 1998; Little, 2000). Epidemiological studies of women exposed to ionizing radiation suggest there may be a period of mammary gland development, subsequent to the adolescent proliferative phase but prior to a first pregnancy, during which the mature, quiescent mammary gland is particularly susceptible to γ-irradiation-induced tumorigenesis (Land et al., 1980; Aisenberg et al., 1997).
Mammalian cells possess surveillance mechanisms to detect and repair radiation-induced DNA damage, one of the most important being the tumor suppressor gene, TP53 (Levine et al., 1991; Cox and Lane, 1995; Ko and Prives, 1996; Levine, 1997). Functional p53 is not essential for survival to adulthood, but mice homozygous for the Trp53-/- allele show increased incidence of a spectrum of tumors (Donehower et al., 1992). As many as 40% of human breast cancers (Coles et al., 1992) and up to 50% of all cancers exhibit mutations in TP53, making it the most commonly mutated gene in human cancers (Levine et al., 1991). Activated p53 can mediate cell-cycle arrest as well repair of radiation-induced double-strand breaks in DNA (reviewed by Amundson et al., 1998). Post-translational modifications of p53 facilitate its stabilization, nuclear accumulation, and increased half-life (Siliciano et al., 1997), promoting transcription of downstream target genes including p21/WAF1 and Mdm2.

Biological consequences of p53 activity include cell cycle arrest, induction of senescence, or apoptosis, depending upon what additional signals are received and within what context. Studies of both embryonic and adult murine tissues have demonstrated tight regulation of Trp53 activation following γ-irradiation, and suggest that these responses are not only cell type-specific, but may also be developmentally regulated (Merritt et al., 1994; Midgley et al., 1995; MacCallum et al., 1996).

Despite the critical role p53 plays in suppressing radiation-initiated transformation, and studies suggesting the mature, quiescent breast is particularly sensitive to radiation-induced tumorigenesis, little is known about p53 responses to ionizing radiation in the adult mammary gland across its post-natal developmental spectrum. Here we show that p53-mediated responses to γ-irradiation in mammary epithelial cells (MECs) vary during post-adolescent mammary gland development, and that these responses are influenced by the degree of proliferation intrinsic to specific developmental stages, not by the accompanying level of differentiation. Furthermore, we demonstrate that MECs stimulated to proliferate in the presence or absence of steroid hormones show equivalent p53-mediated responses to ionizing radiation. Together these data suggest that radiation-induced p53 activation is greatest during peak proliferative stages of mammary gland development and may be mediated in vivo by the mitogenic actions of steroid hormones.

MATERIALS AND METHODS
Animal and tissue procedures
Fourth inguinal mammary glands were isolated from age-matched 8- to 12-week old virgin (V), day-4 pregnant (P4), day-15 pregnant (P15), day-5 lactating (L5) and day-7 weaning (W7) BALB/c-TP53+/- and -Trp53 -/- female mice before, or 6 hours after treatment with 5 Gy of whole body irradiation (n=3 per developmental stage/genotype/radiation group). Day of gestation was determined by counting forward from the appearance of a vaginal plug, which was counted as day one. Radiation was administered using a 137Cs irradiator. Six hours after γ-irradiation, tissues were fixed overnight in 10% phosphate-buffered formalin (PBF), rinsed in 70% ethanol, and paraffin-embedded.

Immunohistochemistry
For detection of p53 and p21/WAF1, 4 μm sections of mammary tissue were deparaffinized in xylene, rehydrated in graded ethanol, rinsed in phosphate-buffered saline (PBS) then subjected to microwave antigen retrieval in 0.1M citrate buffer. Tissues were incubated in 0.3% hydrogen peroxide (Sigma Chemicals, St. Louis, MO) in methanol, blocked with 5% nuclear acid blocker (Roche Diagnostics Corp., Indianapolis, IN) in 0.1 M maleate buffer, and incubated overnight at 4°C with rabbit polyclonal CM5 anti-p53 (1:1500, Novacastra, Newcastle upon Tyne, UK) or rabbit polyclonal Ab-5 anti-p21/WAF1 antiserver (1:50, Oncogene Research Products, Cambridge, MA). Negative controls for p53 immunohistochemistry consisted of mammary tissue from age- and treatment-matched Trp53 -/- female mice. Omitting primary antibody from staining procedures generated negative controls for p21/WAF1 and proliferating cell nuclear antigen (PCNA) immunohistochemistry. The mouse monoclonal PC10 anti-PCNA antibody (1:50, BD Pharmingen, San Diego, CA) and the MOM Detection Kit (Vector Laboratories, Burlingame, CA) were used according to the manufacturer’s directions to detect PCNA expression in 4 μm sections of mammary tissue that had been subjected to microwave antigen retrieval. Immunolabeled complexes were amplified using the Vector ABC Elite avidin-biotin-horseradish peroxidase labeling kit (Vector Laboratories) and 3,3’-diaminobenzidine (Sigma). Tissue sections were counterstained with Methyl Green, dehydrated through graded ethanol and xylene, and cover-slipped. The percentage of cells cycling was determined using the BioQuant Imaging Software System (R&M Biometrics, Inc., Nashville, TN). For in vivo glands, a minimum of 1500 mammary epithelial cells was counted (3 animals per treatment/developmental stage, 6-8 fields/animal). For whole organ cultures, a minimum of 1200 mammary epithelial cells was counted per treatment (600 cells per culture/duplicate cultures per treatment). Differences in the percentage of cells cycling between treatments were determined using a test of 2 proportions with a 95% confidence interval.

In situ end-labeling of DNA
Four μm sections of mammary tissue were deparaffinized, incubated in 0.3% hydrogen peroxide in methanol, and subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) of apoptotic DNA, using the FragEL DNA Fragmentation Kit (Oncogene Research Products). Sections were counterstained with Methyl Green and the percentage of apoptotic cells was determined using the BioQuant Imaging Software System. For in vivo glands, a minimum of 2400 mammary epithelial cells and 10,000 lymphocytes from intramammary lymph nodes were counted per treatment/per developmental stage (3 animals per treatment/developmental stage, 2-4 fields/animal). For whole organ cultures, a minimum of 1200 mammary epithelial cells was counted per treatment (600 cells per culture/duplicate cultures per treatment). Differences in the percentage of TUNEL-positive cells among treatments were compared using a test of 2 proportions with a 95% confidence interval.

Northern blot hybridization
Fourth inguinal mammary glands from unirradiated V, P4, P15, L5 and W7 BALB/c-TP53+/- female mice were homogenized in Ultraspec RNA (BioTecx Laboratories, Houston, TX), 1 ml/100 mg of tissue, before RNA isolation using a modified Chomczynski method (Chomczynski and Sacchi, 1987). Ten μg of total RNA was separated on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane (Zetabind; CUNO, Meriden, CT) and hybridized with a 32P-labeled β-casein cDNA probe. The blot was then stripped and reprobed with a 32P-labeled GAPDH cDNA probe to demonstrate equivalent sample loading.

Whole organ cultures
Fourth inguinal mammary glands from age-matched, unprimed, 8- to 12-week old virgin BALB/c-TP53+/- and -Trp53 -/- female mice were removed aseptically, cut in half then floated on siliconized lens paper
Cell cycling predicts p53 responsiveness

Control medium consisted of serum-free DMEM/F12 buffered with Hepes and NaHCO₃, plus insulin (5 μg/ml; GibcoBRL Life Technologies, Grand Island, NY). Hormone-supplemented medium was prepared by adding 1 ng/ml 17β-estradiol (Sigma) and 1 μg/ml progesterone (Sigma) to control medium. Growth factor (GF)-supplemented medium was prepared by adding 20 ng/ml each of EGF (Sigma) and TGFα (R&D Systems, Minneapolis, MN) to control medium. Whole organ cultures (WOCs) were maintained for 96 hours in 5% CO₂ in air, with daily media changes. After 96 hours, WOCs were subjected to 5 Gy of γ-radiation, using a 137 Cs irradiator, while control WOCs were sham irradiated.

RESULTS

Nuclear p53 is differentially expressed in the post-natal mammary gland following γ-irradiation

We recently demonstrated that virgin mammary glands of Trp53+/+ mice fail to mount p53-mediated responses to γ-irradiation, but upon transient treatment with pregnancy-associated hormones, a robust radiation-induced p53 response is observed (Kuperwasser et al., 2000). This suggested that radiation-induced p53 responses might be developmentally regulated in the post-natal mammary gland. To examine this possibility, we treated age-matched virgin (V), mid-pregnant (P15), lactating (L5) and weaning (W7) Trp53+/+ and Trp53−/− female mice with 5 Gy of whole body γ-radiation and compared their p53 responses to that of unirradiated mice using immunohistochemical approaches. Pilot studies of radiation responses at 10 and 30 minutes, and 1, 6 and 24 hours post-irradiation determined the 6-hour timepoint to be optimal for multi-parameter measurements. While nuclear p53 was detectable 1 hour following irradiation, p21/WAF1 expression peaked at 6 hours, consistent with the kinetics of the p53 radiation response (MacCallum et al., 1996) (C. Kuperwasser, unpublished results). Additionally, TUNEL staining performed at early timepoints detects double-strand DNA breaks still undergoing repair that, by 6 hours post-irradiation, are the hallmark of apoptotic cells. Furthermore, analysis at 24 hours post-irradiation did not show significant increases in cell death in irradiated virgin glands (data not shown), ruling out a delayed induction of apoptosis as a mechanism for the differential apoptotic responses observed across the developmental spectrum.

Nuclear accumulation of p53 is requisite for its biochemical and biological activities (Martinez et al., 1997; Middeler et al., 1997). In unirradiated control Trp53+/+ female mice, nuclear p53 was not detected by immunohistochemical staining in the epithelium of mammary glands at the four stages of post-natal mammary gland development analyzed (Fig. 1A-D). In
contrast, following γ-irradiation of Trp53+/+ female mice, robust nuclear p53 staining was observed in many ductal epithelial cells of P15 glands (Fig. 1F), while in W7 tissues strong nuclear staining was seen in a moderate number of ductal epithelial cells (Fig. 1H). However, irradiated V and L5 glands showed little or no nuclear p53 staining in ductal epithelium (Fig. 1E,G), stromal adipocytes, or periductal fibroblasts. Nuclear p53 could be seen in irradiated lymphocytes of the intramammary lymph node at all stages of development (data not shown) and was used as an internal control verifying radiation delivery.

Non-specific staining or cross-reactivity of the CM5 antibody with nuclear proteins can be excluded since irradiated mammary epithelium from age- and developmental stage-matched Balb/c-Trp53−/− mice were devoid of nuclear p53 staining (Fig. 1I-L). These data show that expression of radiation-induced nuclear p53 varies in a cell type-specific manner within the mammary gland, and within the epithelial compartment of the gland, nuclear p53 expression varies across the developmental spectrum.

**Nuclear localization of p53 correlates with transactivation of its target gene, p21WAF1**

Transcriptional activity of p53 following γ-irradiation can be measured by induction of downstream target genes, including p21WAF1, Bax and GADD45 (el-Deiry, 1998; Bouvard, 2000). We sought to determine the biochemical activity of nuclear p53 by assessing its ability to transactivate p21WAF1, as its induction in the mammary gland was shown to be p53 dependent (Jerry et al., 1998). Basal levels of p21WAF1 protein expression were uniformly low in mammary tissues of unirradiated control Trp53+/+ (Fig. 2A-D) and Trp53−/− female mice (data not shown), regardless of developmental stage. However, following irradiation, strong nuclear staining of p21WAF1 was observed in many ductal epithelial cells of Trp53+/+ P15 mammary glands and also at lower frequency in W7 glands (Fig. 2F,H). Lymphocytes of intramammary lymph nodes also showed robust nuclear p21WAF1 staining (data not shown). Mammary epithelium from Trp53+/+ V and L5 glands exhibited little to no nuclear p21WAF1 staining (Fig. 2E,G), though nuclear p21WAF1 staining was evident in the irradiated lymphocytes of their intramammary lymph nodes at levels comparable to P15 and W7 mammary tissues (data not shown). Consistent with their lack of nuclear p53 expression, stromal adipocytes and periductal fibroblasts failed to show significant nuclear p21WAF1 staining at any stage of development in control or irradiated wild-type tissues.

Mammary tissues from irradiated Trp53−/− mice were devoid of nuclear p21WAF1 expression at all stages of development (Fig. 2I-L), demonstrating the p53-dependent nature of the
response in Trp53+/+ mice. Thus, γ-radiation-induced transcriptional activity of p53 varies during post-natal mammary gland development, and parallels its nuclear localization.

**Activated p53 mediates biological responses at distinct stages of development**

Cell cycle arrest and apoptosis are biological responses to γ-irradiation mediated by p53 in a variety of cell types (Kastan et al., 1991; Lowe et al., 1993a; MacCallum et al., 1996; Meyer et al., 1999; Komarova et al., 2000; D’Sa-Eipper et al., 2001). Basal apoptotic indices in mammary epithelium from unirradiated control Trp53+/+ and Trp53−/− female mice were less than 1% at each of the four stages of post-natal mammary gland development examined, consistent with previously published results (Meyn et al., 1996; Kuperwasser et al., 2000), while levels of apoptosis in the mammary epithelium of irradiated Trp53+/+ mice varied with stage of development and closely paralleled the expression of nuclear p53 (Fig. 3). In the irradiated V gland, there was a small but significant increase in epithelial cell apoptosis above basal levels (3.4% vs. 0.92%, P<0.05) while no increase was seen in L5 mammary tissue (0.82% vs. 0.37%, P>0.05). In contrast, a robust and significant increase in apoptosis was seen in the irradiated epithelium of the P15 gland (11.73% vs. 0.55%, P<0.05). The irradiated W7 gland displayed an intermediate level of apoptosis (4.69% vs. 0.57%, P<0.05), significantly higher than basal levels but lower than mid-pregnant values. Unlike the mammary epithelial cell compartment, the percentage of apoptotic lymphocytes in the intramammary lymph nodes of irradiated Trp53+/+ mammary glands remained consistently high regardless of developmental stage (mean=20.7%±5.64%).

The mammary epithelium of irradiated Trp53+/− female mice showed a distinct absence of cell death, as did lymphocytes of intramammary lymph nodes at all stages of development examined (data not shown), confirming the essential role of p53 in radiation-induced apoptosis in these tissues. Therefore, as with its biochemical activity, the biological activity of p53 in the mammary gland varies with the developmental stage of the gland and is cell-type specific.

**Proliferation, not differentiation, influences radiation-induced p53 activity**

Mammary glands from virgin and mid-pregnant mice represent diverse stages of post-natal development, both morphologically and functionally. Specifically, the mid-pregnancy (P15) gland exhibits a significantly increased proliferative capacity as it responds to the hormonal milieu of pregnancy. Furthermore, by day 15 of pregnancy, the mammary epithelium has initiated the genetic program that drives it toward its fully differentiated, lactating state (Robinson et al., 1995). We sought to distinguish which of these, proliferation or differentiation, might influence p53 response to γ-irradiation, and therefore chose to examine radiation-induced p53 responses in mammary glands of 4-day pregnant (P4) mice, which show increased proliferation, but an absence of differentiation.

To quantify the degree of stage-specific proliferation at the time of irradiation, we immunolabeled proliferating cell nuclear antigen (PCNA) in control and irradiated mammary tissues of virgin, P4 and P15 mice. In non-quiescent cells, PCNA can be detected during all phases of the cell cycle, but its expression increases during S-phase (Celis and Celis, 1985). In contrast, BrdU will only be incorporated into cells that successfully transit the S-phase. Thus, to use BrdU labeling as a means of identifying cycling cells would potentially eliminate from quantitation those cells arresting at the G1/S checkpoint, as well as cells in G2 during the radiation event and subsequently arresting at G2/M, both points at which p53 has been shown to mediate cell cycle-arrest (Kastan et al., 1991; Yonish-Rouach et al., 1993; Morgan and Kastan, 1997). Quantitation of PCNA, therefore, reflects a more inclusive profile of cycling mammary epithelial cells. The percentage of PCNA-positive cells in the V mammary gland was significantly lower than either the P4 or P15 gland (5.86% vs 18.4% and 9.46%, respectively, P<0.05; Fig. 4A,Bi-iii). Additionally, there is strong correlation between the percentage of cycling cells and the percentage of apoptotic cells in the irradiated epithelium of V, P4 and P15 glands, but not in unirradiated control glands (Fig. 4A,Biv-vi; data not shown).

Our data, therefore, confirm the proliferative burst intrinsic to the P4 mammary gland and induced by the hormonal stimuli of early pregnancy. Following γ-irradiation, and similar to the P15 gland, the epithelium of the P4 mammary gland shows strong induction of nuclear p53, as well as nuclear p21/WAF1 (Fig. 5Aiii,iv). Yet unlike the P15 developmental stage, the P4 gland maintains its undifferentiated state as measured by induction of β-casein mRNA (Fig. 5B), the first lactation-essential milk protein to be upregulated during pregnancy (Robinson et al., 1995). Together, these data suggest that p53-mediated responses to γ-irradiation are greatest in the proliferating epithelium of the mammary gland, and the
robustness of the responses seen is dictated by the extent of proliferation, not differentiation.

**Proliferative stimuli render p53 competent to respond to γ-irradiation**

In vivo, proliferation in the mammary gland is intimately influenced by steroid hormones. Studies suggest this action is indirect or paracrine in nature, and may be mediated by the mitogenic effects of growth factors released by epithelial and stromal cells in response to steroid hormones (Woodward et al., 1998). As such, we sought to distinguish direct hormonal modulation of p53 activity from any indirect effects on p53 activation, as mediated by the proliferative actions of steroid hormones. To do so, we induced the epithelial cell compartment of whole organ cultures (WOCs) to proliferate in the presence or absence of the combined steroid hormones, estrogen plus progesterone, then irradiated the glands and measured p53-dependent responses. WOCs cultured in the presence of estrogen (1 ng/ml) plus progesterone (1 μg/ml) but without growth factors, or in the presence of EGF (20 ng/ml) plus TGFα (20 ng/ml) but without steroid hormones, showed small but significant increases in the percentage of cells cycling (3.57% and 3.45%, respectively; P<0.05) compared to control glands grown in the absence of both hormones and growth factors (0.98%). These increases were also consistent with previous studies (Spitzer et al., 1995). After treatment with 5 Gy of γ-radiation, nuclear localization of p53 and p21/WAF1 could be detected in luminal epithelial cells of WOCs maintained in the presence of either hormones or growth factors (Fig. 6B,C and E,F, respectively), but not in those grown in control medium (Fig. 6A,D). Additionally, the percentage of apoptotic cells seen in irradiated WOCs cultured...
in either hormone- or growth factor-supplemented medium was significantly higher than irradiated control WOCs, and correlated closely with the percentage of cycling cells under these growth conditions (Fig. 7). Thus, induction of a p53-dependent response to γ-irradiation in MECs from unprimed virgin mice could be achieved using growth factors to stimulate their proliferation prior to irradiation. These data suggest that direct hormonal modulation of p53 is not required to activate p53 in response to ionizing radiation.

**DISCUSSION**

**Radiation-induced p53 activity varies during postnatal development**

Our initial set of experiments revealed an interesting and somewhat unexpected profile of p53-mediated responses to γ-irradiation. Nuclear localization of p53 protein correlated with its biochemical activity, and this correlation could be demonstrated in both epithelial and lymphocytic cells, which displayed parallel expression/activity profiles. Likewise, significant induction of apoptosis was only seen in stages of mammary gland development that also exhibited accumulation of nuclear p53. These observations are in agreement with previous work showing transcriptional activity of p53 is required to mediate its apoptotic responses (Jiminez et al., 2000; Zhu et al., 1998). Additionally, our evidence that nuclear p53 accumulates in some cell types within the heterogeneous mammary gland but not others, is also consistent with earlier studies demonstrating cell type-specific induction of p53 in response to γ-irradiation (Midgley et al., 1995).

Surprisingly, the p53 responses to γ-irradiation exhibited by mammary epithelial cells differed dramatically across the post-natal developmental spectrum. Despite the fact that these cells are intrinsically similar, there appear to be sufficient differences in ontogeny to significantly alter nuclear accumulation of p53 and its concomitant functional activity following γ-irradiation. While unexpected, this is not entirely without precedence, as MacCallum et al. (MacCallum et al., 1996) also demonstrated developmentally regulated differences in tissue-specific p53-mediated radiation responses.

The data presented here confirm and extend the work of Kuperwasser et al. (Kuperwasser et al., 2000) demonstrating attenuated p53 responses to γ-irradiation in the mammary epithelium of Trp53+/+ virgin mice. In addition, the present study demonstrates that strong induction of p53 in the mammary epithelium of irradiated mid-pregnant Trp53+/+ mice is associated with significant increases in apoptosis. In some radiation-sensitive tissues such as splenic white pulp and the bases of the crypts in the small intestine, the presence of high levels of Trp53 mRNA have been suggested as a factor influencing radiation-induced apoptotic responses (Komarova et al., 2000). Unlike these tissues, however, the differential accumulation of nuclear p53 detected in irradiated P15 glands cannot be attributed to differences in Trp53 mRNA levels between virgin and mid-pregnant stages, since the presence of similarly high levels of Trp53 transcript in glands of virgin, mid-pregnant and weaning females has been previously demonstrated (Pinkas and Jerry, unpublished results) (Jerry et al., 1998; Kuperwasser et al., 2000). It is doubtful, therefore, that the observed p53-mediated responses to radiation in the mammary gland are modulated at the transcriptional level.

As a consequence of cell death, apoptotic cells are identified and efficiently removed by phagocytes (reviewed by Fadok and Chimini, 2001). Because the underlying principle of TUNEL labeling precludes its use as an early indicator of apoptosis, accelerated clearance of apoptotic cells in V and L5 glands cannot be entirely ruled out. However, studies on the kinetics of apoptotic cell clearance show that while the minimum amount of time needed for clearance may be several hours, DNA fragmentation, such as detected by TUNEL staining, can precede clearance by up to several days (Blaschke et al., 1996; Pompeiano et al., 1998), suggesting that rapid clearance is an unlikely explanation for the disparate levels of apoptosis observed during mammary gland development.

At the protein level, mutation and nuclear exclusion have both been shown to result in compromised p53 activity. Cytoplasmic sequestration of p53 in mammary epithelial cells has been postulated as one mechanism that may promote breast...
tumorigenesis (Moll et al., 1992; Kuperwasser et al., 2000), however, unlike the findings of Kuperwasser et al., cytoplasmic p53 in mammary epithelium from control or irradiated \(Trp53^{+/+}\) virgin mice was not detected in this study. The reasons for this inconsistency are unclear, but it may reflect intrinsic lot-to-lot variability of epitopes in polyclonal antisera from different animals, and the significantly lower antibody concentrations used in our studies (1:1500 vs. 1:200). However, in agreement with Kuperwasser’s results, no nuclear p53 or p21/WAF1 was detected, nor was significant induction of apoptosis observed in the mammary epithelial cells of irradiated virgin \(Trp53^{+/+}\) mice.

This study not only confirms that the mammary gland of virgin mice represents a stage of post-natal development characterized by attenuated p53 responses to ionizing radiation, but also reveals that the lactating gland is similarly refractory. In contrast, glands of pregnant and weaning mice show significant p53-mediated responses to ionizing radiation.

**Fig. 6.** p53 and p21/WAF1 expression are induced in proliferating, \(\gamma\)-irradiated whole organ cultures. Immunohistochemistry using CM5 anti-p53 (A-C) and Ab5 anti-p21/WAF1 (D-F) antisera was performed on whole organ cultures maintained for 96 hours in unsupplemented (untreated) medium (A,D), hormone-supplemented (B,E) or growth factor-supplemented (C,F) medium and 6 hours after treatment with 5 Gy of \(\gamma\)-radiation. Hormone-supplemented medium contained estrogen (1 ng/ml) plus progesterone (1 \(\mu\)g/ml), while growth factor-supplemented medium contained EGF (20 ng/ml) plus TGF\(\alpha\) (20 ng/ml). (Magnification 40\(\times\).)

**Apoptotic response to \(\gamma\)-irradiation correlates with extent of epithelial cell proliferation**

During early embryogenesis, many rapidly proliferating tissues have been shown to accumulate high levels of p53 protein following \(\gamma\)-irradiation, without undergoing apoptosis, suggesting p53 may be functioning to prevent teratogenesis and preserve developmental plasticity (MacCallum et al., 1996). As gestation proceeds, clear heterogeneity of p53 induction and of p53-mediated apoptotic responses to \(\gamma\)-irradiation become apparent, suggesting that p53 may assume a quite different role in fully developed tissues. In the adult
animal, in the absence of tissue injury or inflammation, homeostatic cellular replacement in tissues remain relatively constant over time (Kumar et al., 2000; Gressner, 2001; Renehan et al., 2001). In many adult tissues, no clear correlation between proliferation and p53-mediated response to irradiation has been demonstrated (MacCallum et al., 1996; Merritt et al., 1994; Midgley et al., 1995). The mature mammary gland is unique, in that it is characterized by defined periods of extensive cellular proliferation and differentiation during pregnancy in response to endocrine stimulation, and this process is recapitulated to a much lesser extent during each estrus cycle (Robinson et al., 1995). In this study, the greatest radiation-induced p53-mediated responses are seen in the early- and mid-pregnancy mammary glands, where the percentage of apoptosis in mammary epithelial cells closely correlates with the percentage of cells cycling. Relative to the quiescent V gland, both the P4 and P15 mammary glands show extensive epithelial cell proliferation (Fig. 4) with the P4 gland not yet displaying the hallmarks of differentiation (Fig. 5B). These results suggest it is the degree of proliferation within the epithelial cell compartment that influences p53-mediated radiation-induced apoptosis in the mammary gland, not the level of epithelial cell differentiation. Using an alternative in vitro approach, stimulating epithelial cell proliferation in whole organ cultures with either steroid hormones or growth factors prior to irradiation, we confirmed the correlation between increased cell cycling and increased p53-mediated apoptotic responses to γ-irradiation. Additionally, this approach revealed that the action of steroid hormones on p53-mediated responses to γ-irradiation in mammary epithelial cells is likely to be indirect, via their mitogenic actions on the epithelium. Taken together, these data suggest that mechanisms intimately associated with proliferation are acting to relieve the functional repression of p53 observed in the quiescent V mammary gland, thus facilitating its activation during periods of increased mammary epithelial cell cycling.

Regulation of p53 activity is exquisitely complex, and there are numerous diverse signals capable of activating it. As such, the correlation between mammary epithelial cell proliferation and radiation-induced p53-mediated apoptosis may be explained by several mechanisms. These include changes in subcellular localization of p53 during the cell cycle, putative phosphorylation of p53 by radiation-responsive kinases that increase its stability and half-life, and potential conflicts in growth/arrest signals arising from the presence of increased levels of proteins required to both drive and halt the cell cycle.

A number of studies have demonstrated cell cycle-dependent, subcellular redistribution of p53, showing that the protein accumulates in the nucleus during late G1 of the cell cycle and is thus poised to act at the G1/S restriction point (Martinez et al., 1991; Shaulsky et al., 1990; Shaulsky et al., 1991). Importantly, additional studies concluded that phosphorylation of amino-terminal serine residues, resulting in increased transcription of p53-dependent genes, Mdm2 and p21WAF1, was compartmentally restricted and required nuclear localization of p53 for phosphorylation to occur (Martinez et al., 1997). Redistribution to the nucleus of cycling cells, places p53 in the proper cellular compartment to be modified by nuclear kinases such as ATM, which itself is activated in response to DNA damage induced by ionizing radiation (Watters et al., 1997). Post-translational modification of p53 by ATM and/or DNA-PK results in phosphorylation of specific serine residues in the amino terminus of the protein, promoting its dissociation from Mdm2, and thus increasing its half-life (Shieh et al., 1997; Mayo et al., 1997). Stabilized p53 may then accumulate in the nucleus, activating transcription of downstream target genes including p21WAF1, GADD45 and Bax, which orchestrate cell-cycle arrest, facilitate DNA repair, and induce apoptosis, respectively. However, p53 has recently been placed in the growth-factor-responsive AKT signaling pathway, which is thought to enhance cellular survival and proliferation by promoting Mdm2-mediated ubiquitination and degradation of p53 (Mayo et al., 2001; Zhou et al., 2001). While this mechanism may aid proliferation, it is not necessarily inconsistent with radiation-induced activation of p53 during stages of rapid cell growth, since cellular responses to radiation result in modification of both Mdm2 and p53 to promote their dissociation and override the negative regulatory effects of Mdm2 (Mayo et al., 1997). Additionally, the tumor suppressor protein, PTEN, recently shown to negatively regulate the AKT pathway, is also upregulated by p53 and, following p53 activation, may act to further protect it from Mdm2-mediated degradation (Mayo et al., 2002).

Previous studies provide further evidence suggesting a link between cell cycling and cell death. Apoptosis can be induced in both mouse and rat embryonic fibroblast cell lines co-expressing E2F1 and p53, suggesting that apoptosis can result from a conflict in growth and arrest signals (Wu and Levine, 1994). Likewise, in transgenic mice over-expressing E2F1, increased apoptosis and decreased epidermal thickness and cellularity were observed on a Trp53+ background compared to their Trp53− counterparts, again suggesting conflicting signals of growth and arrest may be resolved by induction of apoptosis (Pierce et al., 1998). Consistent with the pattern of apoptosis we observed in irradiated cycling mammary epithelial cells of P4 and P15 Trp53+/mice, these data argue strongly in favor of a mechanism of p53-mediated cell death arising from conflicting signals that concomitantly attempt to drive and halt the cell cycle. Studies are currently underway in our laboratory to elucidate the molecular mechanisms responsible for regulating radiation-induced p53-mediated apoptosis in cycling epithelial cells of early- and mid-pregnancy mammary glands that appear to be refractory in the quiescent gland of virgins.

While the pattern of apoptotic responses seen in the γ-irradiated murine mammary gland supports a model of conflicting growth and arrest signals, other potential mechanisms may be mediating the observed responses. Bcl2 mRNA levels have been shown to be high in the virgin mammary gland, relative to levels in the mid-pregnancy gland, while Bax mRNA levels remain fairly constant during post-natal development (J. J., unpublished results). Bcl2 levels could, therefore, provide overriding survival signals in virgin mammary epithelium that are lost as the change in Bcl2/Bax ratio favors Bax, and predisposes the mammary epithelium to undergo radiation-induced apoptosis as the gland progresses through pregnancy. In the V gland, inactive p53, incapable of transactivating downstream targets such as Bax, may act to maintain Bcl2/Bax ratios in favor of Bcl2 and survival signals.
Attenuated p53 function in quiescent mammary epithelial cells may increase susceptibility to radiation-induced tumorigenesis

Epidemiological studies confirm that women exposed to ionizing radiation as a result of the Hiroshima bombing, therapeutic fluoroscopies or chest x-rays (Land et al., 1980), or who received mantle-field irradiation for the treatment of Hodgkin’s disease (Aisenberg et al., 1997), show an increased risk of breast cancer relative to the general population. A unifying corollary of these studies is the inverse risk of cancer with age at time of exposure to radiation. The present study has demonstrated that in the mouse mammary gland, p53 responses to ionizing radiation vary during post-adolescent development, with the quiescent virgin gland exhibiting an attenuated apoptotic response. Decreased p53 response to radiation at this stage of development coincides with the ‘window of susceptibility’ that may exist in humans, and during which the mammary epithelium may be at increased risk of radiation-induced transformation. The implications of this observation are significant, as p53 integrity is crucial in minimizing tumorigenic potential in multiple tissues, including the mammary gland (Lozano and Liu, 1998; Jerry et al., 2000).

Induction of apoptosis in response to DNA damage is a well-documented mechanism by which p53 may suppress tumorigenesis in vivo (Symonds et al., 1994). In fact, many therapeutic strategies target the more rapidly dividing tumor cells with DNA-damaging agents as a means of eliminating them through p53-mediated apoptosis (Lowe et al., 1993b) and abrogation of p53 function can contribute significantly to radio- or chemoresistance (Lowe et al., 1994).

Loss of this protective mechanism may promote the accumulation, rather than the deletion, of cells with severe genetic damage. In the small intestines, but not the colon, cells corresponding to putative stem cells in the hierarchy of the intestinal crypts undergo apoptosis following γ-irradiation, suggesting a mechanistic basis for the decreased incidence of radiation-induced carcinoma in the small intestines compared to the colon (Merritt et al., 1994). Failure of irradiated virgin mammary epithelium to undergo apoptosis may represent a situation comparable to that of the colon, in which putative stem cells incur DNA damage but they are not removed by p53-mediated cell death.

In addition to apoptosis, another important role of p53 is mediating repair of double strand DNA breaks following γ-irradiation. Previous studies have identified rapidly proliferating cell populations as being highly susceptible to radiation-induced transformation (Chan and Little, 1981). However, Ulrich et al. have demonstrated increased genomic instability in irradiated mammary epithelial cells allowed to remain in situ for a period of time following irradiation (Ulrich et al., 1999), suggesting DNA repair is compromised in these cells. These data argue that a population of quiescent cells may be equally susceptible to the effects of radiation-induced genetic instability. Furthermore, previous studies by Ponnaiya et al. (Ponnaiya et al., 1997) have suggested a mechanistic link between instability and radiation-induced cancer.

Therefore, the functional repression of p53 observed in quiescent mammary epithelial cells not only renders them resistant to apoptosis, it may also compromise their ability to detect and repair DNA damage. Though these mechanisms may preserve the integrity of the tissues, they may also allow increased accumulation of mutations. As such, developmental regulation of p53 activity may represent a molecular basis for tumor susceptibility in mammary epithelial cells.

The authors would like to thank Anneke C. Blackburn and David A. Steele for thoughtful discussions and critical reading of the manuscript, and Sharon Marconi for expert technical assistance in processing mammary tissues. This work was supported by a graduate fellowship to L. M. M. from the Massachusetts Department of Public Health (340006670) and grants to D. J. J. from the National Institutes of Health (CA66670, CA87531) and the Massachusetts Department of Public Health (43088PP1017). This material is based on work supported by the Cooperative State Research Extension, Education Service, US Department of Agriculture, Massachusetts Agricultural Experiment Station and Department of Veterinary and Animal Sciences under Project Nos. MAS00821 and MAS00714.

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