Differential Contextual Responses of Normal Human Breast Epithelium to Ionizing Radiation in a Mouse Xenograft Model


Abstract

Radiotherapy is a key treatment option for breast cancer, yet the molecular responses of normal human breast epithelial cells to ionizing radiation are unclear. A murine subcutaneous xenograft model was developed in which nonneoplastic human breast tissue was maintained with the preservation of normal tissue architecture, allowing us to study for the first time the radiation response of normal human breast tissue in situ. Ionizing radiation induced dose-dependent p53 stabilization and p53 phosphorylation, together with the induction of p21(CDKN1A) and apoptosis of normal breast epithelium. Although p53 was stabilized in both luminal and basal cells, induction of Ser392-phosphorylated p53 and p21 was higher in basal cells and varied along the length of the ductal system. Basal breast epithelial cells expressed ΔNp63, which was unchanged on irradiation. Although stromal responses themselves were minimal, the response of normal breast epithelium to ionizing radiation differed according to the stromal setting. We also demonstrated a dose-dependent induction of γ-H2AX foci in epithelial cells that was similarly dependent on the stromal environment and differed between basal and luminal epithelial cells. The intrinsic differences between human mammary cell types in response to in vivo irradiation are consistent with clinical observation that therapeutic ionizing radiation is associated with the development of basal-type breast carcinomas. Furthermore, there may be clinically important stromal–epithelial interactions that influence DNA damage responses in the normal breast. These findings demonstrate highly complex responses of normal human breast epithelium following ionizing radiation exposure and emphasize the importance of studying whole-tissue effects rather than single-cell systems.

Introduction

Adjuvant radiotherapy is commonly used to improve outcomes for patients with early breast cancer (1). Breast conservation (tumor excision followed by breast radiotherapy) is now used in 70% of operable patients and provides comparable survival to mastectomy (1). Indeed, radiotherapy to the residual breast tissue after conservative surgery prevents recurrence both for invasive breast cancer and for ductal carcinoma in situ, a condition that now accounts for 25% of breast cancer diagnoses (1). Advances in radiotherapy for breast cancer include hypofractionation (2), limited fields of radiation exposure (3), and intraoperative radiotherapy (4, 5). The use of radiotherapy, particularly targeted approaches, is likely to increase (6).

Given the importance of radiotherapy in the management of breast cancer, little is known about the mechanistic and dose-limiting effects of radiation upon normal breast tissue, compounded by the considerable heterogeneity of normal tissue responses (7). These differences in tissue responses may result from genetically determined factors that influence cellular radiosensitivity or the microenvironment (7, 8).

The TP53 gene encodes the p53 protein, which integrates multiple cellular stress signals including those induced by radiation and via posttranslational modification (including phosphorylation, acetylation, and methylation of specific residues) triggers growth arrest or programmed cell death (apoptosis) by modulating gene expression (9–11). p53 can also trigger cell death independently of transcription and has widespread effects on the development and normal function of organisms, including effects on cellular senescence, autophagy, and glucose metabolism (12, 13). Cells in vivo demonstrate tissue-specific p53 pathway responses to radiation-induced DNA damage (14–20). Actively proliferating cells within self-renewing tissues show stabilization and transcriptional activation of p53 that leads to apoptosis. In contrast, some nonproliferating tissues show neither p53 stabilization...
nor apoptosis. An intermediate group of nonproliferative tissues shows p53 induction but not apoptosis (18, 19). These tissue-specific p53 pathway responses to ionizing radiation are genotype dependent (14). In the breast, basal and luminal mammary cells propagated and maintained as isolated cells or as small tissue fragments in vitro exhibit distinct responses to ionizing radiation. Basal cells exhibit transitory growth arrest, whereas luminal cells show a more prolonged period of arrest (21). However, these in vitro studies rely on the use of specific culture conditions, which themselves influence radiation responses (22). Thus, in vitro experiments designed to predict normal tissue responses suffer from a variety of artifacts that limit their usefulness (7).

The responses of normal human tissues to ionizing radiation in situ have been studied in skin biopsies from patients receiving radiotherapy for internal tumors such as breast and prostate (23–25), but other models of normal human tissue responses to radiation that preserve tissue architecture and cellular interactions with the stromal environment are currently lacking. The aim of this study was to develop a xenograft model of normal mammary tissues derived from women with breast cancer at the time of surgery (26, 27) and apply the model to investigate the molecular pathways that are activated by ionizing radiation in human mammary tissues in vivo. We sought to test the hypothesis that distinct cell types respond differently to the same radiation dose and that the surrounding stromal context influences these epithelial responses. Our model preserves the anatomic relationships of ductal/lobular units with the surrounding human breast stroma and for the first time permits time-course and dose-response studies of different cell types within their normal 3-dimensional architecture following in vivo irradiation.

**Methods**

Macroscopically, normal breast tissue from 20 consenting patients with primary, previously untreated stage I or stage II breast carcinoma was excised from the breast adjacent to the excision cavity at the time of wide local excision. Tissues were transported in a sterile container, stripped of excess fat, divided into 2 × 1 × 1-mm³ fragments and placed in Dulbecco’s modified Eagle’s medium until implantation. Developing and extending previous methods that maintain long-term tissue viability and the anatomic relationships of ductal/lobular units (26, 27), breast tissue fragments were implanted under general anesthesia subcutaneously into the flanks of 6 to 12 SCID mice per patient and used to study the molecular pathways activated in response to ionizing radiation in vivo.

Mice were housed under normal conditions, and 4 days later, whole-body γ-irradiation from a CIS Bio International 637-cesium source was delivered at a metered dose of 0 gray (Gy) (controls) and 2 or 5 Gy to duplicate animals (dose rate = 0.4 Gy/min) for each patient sample. The xenografted human tissues were harvested 4 hours after radiation exposure, for which previous in vivo studies have shown near maximal stabilization of p53 together with the activation of downstream signaling pathways and induction of apoptosis (14, 18). In 2 patients, sufficient mammary tissue was available to allow additional comparison of 1- and 4-hour time points after exposure to either 2 or 5 Gy. Sections of formalin-fixed, paraffin-embedded human tissues were stained with hematoxylin and eosin for initial histopathologic examination to confirm the presence of normal breast tissue.

All experiments were performed with permission of the Local (Human) Research Ethics Committee and Animal Licensing Authorities under strict codes of aseptic practice. Xenograft studies were carried out in accordance with the UKCCCR guidelines.

**Immunohistochemistry**

Sections (4 μm) were stained with an avidin–biotin complex peroxidase technique (Vector Elite reagents; Vector Laboratories Ltd, Peterborough, UK) according to the manufacturer’s instructions and detected with diaminobenzidine containing 5 mmol/L of imidazole. Antigen retrieval was performed by boiling for 15 minutes in 10 mmol/L of citrate, pH 6.0, except for γ-H2AX staining for which antigen retrieval was not performed (Table 1). Sections were counterstained with hematoxylin and viewed under a light microscope. For p53, phospho-p53, p21, and Mdm2, staining was quantified using the histoscore, for which staining intensity is divided into 5 groups (0–4, where 0 is negative and 4 is very strongly stained).

### Table 1. Primary antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Species</th>
<th>Dilution</th>
<th>Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>CM1, D. Lane</td>
<td>Rabbit</td>
<td>1/100,000</td>
<td>Citrate</td>
</tr>
<tr>
<td>phospho-p53(Ser15)</td>
<td>9284, Cell Signaling</td>
<td>Rabbit</td>
<td>1/250</td>
<td>Citrate</td>
</tr>
<tr>
<td>phospho-p53 (Ser392)</td>
<td>FP3.1, B. Vojtesek (53)</td>
<td>Mouse</td>
<td>1/10,000</td>
<td>Citrate</td>
</tr>
<tr>
<td>ΔNp63</td>
<td>in-house (54)</td>
<td>Rabbit</td>
<td>1/20,000</td>
<td>Citrate</td>
</tr>
<tr>
<td>TAp63</td>
<td>in-house (54)</td>
<td>Rabbit</td>
<td>1/20,000</td>
<td>Citrate</td>
</tr>
<tr>
<td>p21(1CDKN1A)</td>
<td>Clone 118</td>
<td>Mouse</td>
<td>1/200</td>
<td>Citrate</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Clone 2A10</td>
<td>Mouse</td>
<td>1/100</td>
<td>Citrate</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>9718, Cell Signaling</td>
<td>Rabbit</td>
<td>1/100</td>
<td>None</td>
</tr>
<tr>
<td>Rad51</td>
<td>Ab-1, Calbiochem</td>
<td>Rabbit</td>
<td>1/10,000</td>
<td>Citrate</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>9664, Cell Signaling</td>
<td>Rabbit</td>
<td>1/1,000</td>
<td>Citrate</td>
</tr>
<tr>
<td>HIF1α</td>
<td>H1α67, Novus Biologicals</td>
<td>Mouse</td>
<td>0.1 μg/mL</td>
<td>Citrate</td>
</tr>
</tbody>
</table>
and the percentage of cells in each category is multiplied by the category number and values added together, providing a continuous score that ranges from 0 to 400 (28). For measurements of apoptosis, the percentages of cells stained for activated caspase-3 were recorded.

**Results**

**Tissue procurement**

Staining with hematoxylin and eosin showed that normal breast epithelial and stromal histologic architecture was preserved in at least 1 of 2 duplicate xenografts from each dose and time point in tissue derived from 10 of the 20 patients. The remaining patient samples were excluded, as not all dose or time points contained sufficient breast epithelium and/or the implanted tissues showed poor morphology. As a second screening tool, all samples that contained sufficient epithelium were stained for HIF1α to identify areas of xenografts with suboptimal oxygenation, seen in focal areas of individual tissue fragments in 20% of individual samples. Because all of these samples contained sufficient epithelium in other areas of the section that were not stained for HIF1α, no additional sections had to be excluded from analysis, but all regions of individual tissue fragments with HIF1α staining in control or irradiated mice were considered hypoxic and these specific areas were therefore excluded from further analysis. Indeed, nuclear HIF1α was associated with weak staining for p53 in sham-irradiated animals, in keeping with the concept that hypoxia is an inducer of p53 stabilization (11).

**Cell type and dose response**

Xenograft material from 10 patients was successfully examined for dose-dependent radiation responses at 4 hours post-irradiation. Staining for p53 was exclusively nuclear, and cytoplasmic staining was not seen. Immunostaining demonstrated a clear dose-dependent increase in p53 in xenografts 2- or 5-Gy irradiation compared with control tissues (Table 2). Responses to irradiation were prominent in breast epithelium compared with a minimal response in breast stromal cells (Fig. 1), even though the dose received by each cell in the tissue was the same. A quantitative analysis demonstrated the dose-dependent induction and stabilization of p53, together with p53 phosphorylation at Ser15 and Ser392, induction of p21 (CDKN1A), and induction of Mdm2 (Table 2 and Fig. 2). Apoptosis, measured by the cleavage of caspase-3, also showed a dose-dependent increase. These data also showed considerable heterogeneity of response, demonstrated by the variable p53 response in tissues from individual patients (Supplementary Fig. S1; note also the range of values in Table 2). Staining for ΔNp63 was seen only in basal epithelium and was unaltered by irradiation. TAp63 staining was not seen in control or irradiated tissues.

To assess radiation-induced DNA damage, γ-H2AX was used as a marker of DNA double-strand breaks and Rad51 as a marker of repair by homologous recombination. In sham-irradiated tissues, the number of cells showing γ-H2AX foci was extremely low and each positive cell showed only 1 or 2 foci. After 2 and 5-Gy exposure, both the percentage of cells with γ-H2AX foci and the number of foci in each cell was increased in a dose-dependent manner, although the response was not linear and the higher dose led to a much higher level of residual double-strand DNA breaks in a small percentage of cells (Fig. 3). As with the other endpoints measured, there was considerable heterogeneity of response between different patients (note the range of values in different patients in Table 2). γ-H2AX foci were not prominent in stromal cells in any patient at either dose. No Rad51 foci were demonstrated in breast epithelium at either dose, although we have identified Rad51 foci in xenografted tumor cell lines and in irradiated murine hemopoietic cells, using this antibody (unpublished data).

**The epithelial response depends on cell type and position in the duct**

*In vitro* studies have shown that myoepithelial cells and ductal cells respond differently to ionizing radiation (19).
Consistent with this, the endpoints measured in this study showed a distinct pattern of staining within the ductal epithelium. Most notably, there was a marked variability of staining intensity for p53, phospho-p53, and p21 between basally located and luminal cells within some ducts, as shown in Figure 4. In addition, staining was dependent on the position along the ductal system, with more intense staining in terminal duct lobular units that are most distal from the nipple than in subsegmental ducts that are closer to the nipple (Supplementary Fig. S2).

Time-course studies
Comparison of 1 and 4 hours after exposure to either 2 or 5 Gy showed a similar dose-dependent increase in p53 and phospho-p53 staining in ductal epithelium, although the absolute levels achieved at 1 hour were less than those achieved at 4 hours, results are consistent with those obtained from other studies investigating these effects both in vivo and in vitro (14, 21). In addition, there was no increase in activated caspase-3 staining at 1 hour. Responses were minimal in stromal cells 1 hour postirradiation. This earlier time point allowed us to study the accumulation of γ-H2AX foci, which are rapidly formed at the sites of double-strand DNA breaks and are lost as the breaks are repaired, usually within a few hours (29). Foci of γ-H2AX were readily apparent 1 hour after 2- or 5-Gy exposure, with a higher number of foci visible after the higher dose, as expected (Fig. 3). Unexpectedly, γ-H2AX levels were highly variable in different parts of the same fragments of xenografted tissue. Epithelial cells surrounded by an adipocyte-rich stroma showed more intense staining than cells embedded within a fibroblast-rich stroma (Fig. 5): 12.96% of epithelial cells showed more than 10 foci in fibroblast-rich areas compared with 71.42% of cells in adipocyte-rich areas 1 hour postirradiation. In addition, γ-H2AX was more pronounced in luminal epithelial cells than in basally located cells (Fig. 5).

Discussion
Radiotherapy is commonly used in patients with early-stage breast cancer, but the precise effects of radiation on the normal breast are unknown. We report the development and first use of a xenograft model of normal human breast tissue to study the differential responses of human mammary tissue components to ionizing radiation in vivo. The xenografting method has been demonstrated to provide viable tissues for at least 2 months postengraftment (26, 30) and building on this, our study demonstrates both dose-dependent and time-dependent induction of p53 responses in human breast epithelium. In contrast, the p53 network responses to ionizing radiation previously reported in murine rather than human breast epithelium in vivo initially suggested cytoplasmic sequestration of p53 in the normal, untreated epithelium.
(not identified in the human epithelium here) and translocation of p53 to the nucleus after irradiation in the presence of placental hormones (31). In subsequent studies, cytoplasmic p53 was not seen but nuclear accumulation after irradiation was associated with the proliferation of epithelial cells and accompanied by both growth arrest and apoptosis (32). In other murine studies, exogenous hormonal treatment (absent in the xenograft model reported here) was, by itself, shown to induce p53 nuclear accumulation. Genotoxic agents induced further accumulation of p53 and p21 (33).

In the current study, we have shown differential responses of human mammary epithelium to therapeutically relevant doses of ionizing radiation. The system maintains the architecture of the human mammary gland and allows interactions between the breast epithelium and breast stroma. Human mammary epithelial cells showed a robust and dose-dependent induction of p53 and apoptosis in the first 4 hours following irradiation. The duration and long-term consequences of this induction have not been addressed in this model but are in keeping with the observed persistent increased expression of p53 even 2 to 5 years after radiotherapy in the `normal' human breast (34). The responses identified in the breast epithelium are typical of self-renewing tissues and are characteristically not induced in nonproliferating cells and tissues (14, 17–19), indicating that breast epithelial cells are a distinct tissue type with respect to radiation responses, and their response may reflect the requirement to maintain proliferative potential even if the epithelium is not actively proliferating at the time of irradiation.

Upstream signals influencing p53 activation include phosphorylation of both the N-terminal serine-15 site, largely mediated by ataxia telangiectasia mutated (ATM), and at the C-terminal serine-392 site involved in enhancing DNA binding (9). Although ATM is redundant for p53 induction and...
apoptosis in some epithelial cell types such as hair follicles and small intestine (35), our data indicate that p53 Ser15 phosphorylation is important in mammary epithelium and are consistent with the role of ATM in susceptibility to breast cancer (36). We also demonstrate p53 phosphorylation at Ser392, a stress-specific phosphorylation event that, some suggest, may not be induced by ionizing radiation (9). This discrepancy may indicate a cell-type and tissue-context specificity of phosphorylation. A lack of phosphorylation at Ser392 may be important in breast cancer pathogenesis and inability to phosphorylate p53 at this site has been related to treatment response (37).

Although p53 activation was identified in tissue from every patient, the responses in different cell types within an individual were disparate and interindividual variations were noted (Table 2, Figs. 2–5 and Supplementary Figs. S1 and S2). For example, p53 induction was seen in both myoepithelial (basal) and luminal cells, but the phosphorylation of Ser392 and Ser15 was often more prominent in the myoepithelium, indicating cell-type specificity of radiation response, as noted in vitro (21). There were also clear differences in the induction of p21, which was seen more commonly in basally located cells than in luminal cells. That different cell types in the mammary gland respond differently to ionizing radiation is compatible with observations that isolated basal breast epithelial cells proceed to transitory cell cycle arrest whereas luminal cells achieve a more durable G2-M arrest (21). Although the underlying mechanisms for these differential responses are not clear, the presence of the N-terminal-truncated ΔNp63 isoform in basal but not in luminal cells is expected to play a role. The ΔNp63 isoforms lack the classic p53-family transcription activation domain and therefore act as natural antagonists for p53-family activity and inhibit p53-mediated apoptosis in the skin following UV irradiation (38).

Following UV irradiation, ΔNp63 is degraded by a ubiquitin-dependent process, thereby allowing p53-mediated responses (38, 39). However, we did not see any evidence for degradation of ΔNp63 in basal mammary epithelium following ionizing radiation exposure, suggesting that this protein could continue to inhibit p53 activity in basal cells. Moreover, ΔNp63 upregulates the ATM kinase responsible for the phosphorylation of p53 Ser15 (40) and induces DNA repair genes including Mre11 and BRCA2 (41), suggesting that expression of ΔNp63 by basal mammary cells is, at least, partly responsible for the differences in phosphorylation and response to ionizing radiation of the basal and luminal breast epithelia. The evidence that basal and luminal cells show distinct p53 pathway responses to ionizing radiation is also in keeping with the observation that breast cancer that develops following therapeutic irradiation during breast maturation in young women shows basal-like differentiation (42) either to an ER negative, HER2-positive phenotype or to a triple-negative phenotype. In addition, the observations that p53 mutations are much more common in basal-like tumors than in luminal cancers also imply that these distinct cell types have different dependencies upon p53 (1).

This mouse xenograft model of short-term radiation responses in normal human breast tissues also permitted assessment of double-strand DNA break repair in vivo. We show an expected accumulation of γ-H2AX foci early in the response, with a rapid decline in the number of foci within 4 hours. Although Rad51 foci have been shown both in xenografted tumor cell lines and in irradiated murine hematopoietic cells with the antibody used here (unpublished data), the absence of detectable Rad51 foci in the present study is in keeping with the nonproliferating breast epithelial and stromal cell populations examined and suggests that repair by homologous recombination is not a key pathway in adult breast epithelium.

Stromal components showed minimal responses to ionizing radiation within the time and dose constraints of this model, even though the dose of radiation was the same in all cells in the tissue. Although stromal gene expression may be important in the induction and maintenance of breast cancer (43), we would not anticipate immune or angiogenic responses to be influential in our model, given the short duration of the experiments and the ablation of the immune system in the murine host. Despite the relative lack of response of stromal cells themselves, we observed dramatic differences in the epithelial response depending on the surrounding stromal composition, pointing to a role for the stroma in determining the response of epithelial cell populations. The role of mammary stroma in regulating the epithelium is well appreciated (44, 45), and adipocytes are known to produce estrogen and a variety of growth factors that influence the ductal epithelium (46, 47). Moreover, the association of breast cancer risk with mammary involution during aging may relate to the differences in stromal composition before and after involution, and there is evidence that premalignant changes to breast epithelium are associated with stromal areas high in connective tissue (as in radiologically dense breasts) rather than adipose-rich areas (48, 49). An important implication of our data relating radiation response to the surrounding stromal architecture is that radiation-induced carcinogenesis in human mammary epithelium may vary according to the stroma. These findings emphasize the requirements to study the correct cell type and cellular context in vivo and question the extrapolation of experimental findings using cultured fibroblasts or lymphocytes to predict the response of human epithelial tissues (7, 50).

In conclusion, although data were not obtained for all patients, this xenograft approach allowed examination of the radiation response of normal breast tissues at 2 time points (1 and 4 hours) after 2 doses (2 and 5 Gy) of radiation in the same patient. This model could be used to study the modifying effects of anticancer drugs on radiation responses in vivo or the effect of breast cancer predisposition genes on the sensitivity of normal breast tissue to genotoxic agents, although interpretation of such studies is likely to be challenging in view of the complexities of response we have uncovered here. Nonetheless, the model offers an integrated (systems- and architecture-based) approach and the preservation of normal anatomic relationships of ductal lobular units and their interactions with the original stromal elements may avoid some of the pitfalls inherent to more conventional techniques using cell-based assays (50–52). We
have demonstrated that although breast epithelial cells can be considered to undergo an archetypal p53 pathway response to ionizing radiation exposure, the in vivo responses of individual cell types (basal/luminal), position along the length of the ductal structure, and the epithelial–stromal relationships are more complex than previously supposed and the clinical implications of this deserve further exploration.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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