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Subtype-Specific Radiation Response and Therapeutic Effect of FAS Death Receptor Modulation in Human Breast Cancer

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Abstract

Breast cancer is the most common malignancy diagnosed among women and represents a heterogeneous group of subtypes. Radiation therapy is a critical component of treatment for breast cancer patients. However, little is known about radiation response among these intrinsic subtypes. In previous studies, we identified a significant induction of FAS after irradiation in biologically favorable breast cancer patients and breast cancer cell lines. Here, we expanded our study and investigated radiation response in a mouse model of breast cancer. MCF7 (luminal), HCC1954 (HER2⁺) or SUM159 (basal) cells were implanted orthotopically into the dorsal mammary fat pad of nude mice. These mice were then treated with different doses of radiation to assess tumor growth control. We further investigated the therapeutic effect of FAS modulation by silencing FAS in radiation-responsive tumors and injecting FAS agonist antibody into radiation-resistant tumors. Exposure to radiation inhibited MCF7, and to a lesser extent HCC1954 tumor growth in a dose-dependent manner. In contrast, SUM159 tumors were resistant to radiation. The estimated TCD₅₀ values were 19.3 Gy for MCF7 and 44.9 Gy for SUM159. Radiation induced FAS expression in MCF7 tumors, but not SUM159 tumors. We found that silencing of FAS did not negatively impact radiation response in MCF7 tumors, possibly due to compensation by other apoptotic pathways. On the other hand, FAS activating antibody in combination with radiation treatment delayed SUM159 and HCC1954 tumor growth. However, it did not reach statistical significance compared to radiation treatment alone. Our results suggest that there is intrinsic variation in radiation response among breast cancer subtypes. FAS activation concurrent with radiation slows tumor

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growth in the radiation-resistant subtypes, but the effect was not significant. Alternative subtype-specific modulators of radiation response are under investigation.

INTRODUCTION

Breast cancer is the most common malignancy diagnosed among women worldwide and represents a heterogeneous group of tumors with different molecular features, prognoses and response to therapy (1, 2). Based on distinct gene expression patterns, breast cancers are classified into different subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched and basal-like (3, 4). Luminal A tumors are associated with a low risk of local and distant recurrence, while basal-like tumors, of a similar stage, have higher rates of locoregional failure and worse overall survival (5, 6).

Radiation therapy is an important component of multimodal treatment for women with breast cancer. However, little is known about radiation response among these subtypes. Recent clinical data suggest that distinct patterns may exist in association with each phenotype. In their study of 793 consecutive patients with invasive breast cancer who received breast-conserving therapy, Nguyen *et al.* reported that the five-year local recurrence rate was particularly low for patients with luminal A subtype, while HER2 and basal-like subtypes showed increased rates of local recurrence (7). A similar study performed by Kyndi *et al.* indicated a greater response to radiotherapy in the hormone receptor-positive patients receiving postmastectomy radiotherapy (8). Although the suboptimal rates of local control in the more aggressive tumors may be partially attributable to inherent biologic aggressiveness, additional data from the Early Breast Cancer Trialists' Collaborative Group clearly suggest that estrogen receptor (ER)-positive and ER-negative breast tumor subtypes are distinct in the degree of clinical benefit achieved with radiotherapy (9). Therefore, understanding the intrinsic variation of radiation response in breast cancer subtypes is important in prescribing individualized radiotherapy that maximizes the therapeutic ratio. Furthermore, identification of molecular targets can be used to modulate treatment outcomes in patients with radioresistant tumors.

To study breast cancer subtype-specific radiation responses, our laboratory has analyzed gene expression profiles from a unique cohort of 32 early-stage, biologically favorable (ER⁺) breast cancer patients treated with preoperative radiation as well as 16 biologically diverse breast tumor cell lines. Radiation resulted in marked changes in gene expression in patient tumors and subtype-specific patterns of response in breast tumor cell lines (10). Candidate genes were associated with apoptosis, cell cycle and MAPK signaling pathways. Among these genes, FAS (CD95, APO-1) was consistently and significantly induced after irradiation in the biologically favorable subtypes. FAS is a cell-surface death receptor, which belongs to the tumor-necrosis-factor-receptor family and is able to induce apoptosis when engaging with FAS ligand. Radiation has been shown to upregulate FAS in MCF7 cells with wild-type-p53 activity (11). However, our previously published *in vitro* data also showed that radiation-induced FAS expression in MCF7 and ZR751 breast cancer cells may involve a p53-independent pathway (10). Furthermore, we observed high baseline levels of FAS in a subset of our radioresistant and largely p53 mutant cell lines. In this cohort, a FAS agonist

antibody in conjunction with radiotherapy enhanced radiosensitivity. In the current study, we further evaluated the subtype-specific response to radiation as well as the role of FAS induction in radiation response in a mouse model of breast cancer.

MATERIALS AND METHODS

Animals

Female, 6-to-8-week-old athymic NCr-nu/nu mice (Charles River Laboratories, Frederick, MD or Duke Cancer Institute mouse breeding facility, Durham, NC) were maintained in specific pathogen-free facilities at Duke University Medical Center (Durham, NC). All animal procedures were performed in strict adherence to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University (protocol no. A114-15-04). All surgeries were performed under ketamine/xylazine anesthesia and buprenorphine solution for pain management. The tumors were measured at least three times/week after treatment until they reach 1,500 mm³ or 90 days. Throughout the study, the Duke IACUC Tumor Policy (solid tumors in rodents) was followed. Animals showing any signs of pain or discomfort (loss of normal grooming activity, ruffled hair or self-mutilation) or ulcerated tumors were euthanized immediately. Mice were euthanized by CO₂ asphyxiation or Euthasol[®] injection followed by bilateral thoracotomy as the secondary method.

Cells and Reagents

MCF7 (luminal), HCC1954 (HER2⁺) and SUM159 (basal) human breast cancer cells were purchased from Duke Cell Culture Facility and cultured in DMEM or RPMI media containing 10% fetal bovine serum (FBS, SH30071.03; HyClone[™] Laboratories, Logan, UT) and 1% antibiotic-antimycotic (15240-062; Gibco[®], Grand Island, NY). MCF7 control short hairpin RNA (shRNA) or FAS shRNA knockdown cells were generated as described elsewhere (10) using the shRNA lentiviral vectors (Open Biosystems/Thermo Fisher Scientific[™], Huntsville, AL). The shRNA lentiviral vectors contained a U6 RNA polymerase III-constitutive promoter that drives short hairpin RNA expression. The MCF7 cells were infected with a pool of five human FAS shRNAs (cat. no. 9606102–9606106) or negative control shRNA lentiviral vectors under puromycin (1 µg/ml) selection. Anti-FAS mouse monoclonal antibody CH11 (cat. no. 05–201) was purchased from EMD Millipore (Billerica, MA) and control mouse IgM (clone: MM-30, cat. no. 401601) was purchased from BioLegend[®] Inc. (San Diego, CA).

Clonogenic Survival Assays

MCF7, HCC1954 or SUM159 cells received different doses using the X-RAD 320 irradiator (energy source: X-ray tube; energy: 320 kV; filtration: 2 mm aluminum; and dose rate: 3.464 roentgens/s) (Precision X-ray Inc., North Branford, CT). Media was removed and replaced 24 h postirradiation to provide adequate nutrients for the tumor cells over the 7–14 days required to form colonies. The plates were then washed with PBS, fixed with 10% methanol/10% acetic acid (cat. nos. BDH1135-4LP and BDH3098-3.8LP, respectively; BDH/VWR[®] International LLC, Radnor, PA) and stained with a 0.4% solution of crystal

violet (cat. no. C0775; Sigma-Aldrich® LLC, St. Louis, MO). A ColCount™ colony counter (Oxford Optronix Ltd., Abingdon, UK) was used to image and count the number of colonies per plate, using fixed sensitivity settings so that only colonies of >50 cells were counted. Plating efficiencies (PE) were calculated using the formula: PE = number of colonies/ number of cells seeded, and normalized to the control/sham-irradiated plates. Surviving fraction (SF) was calculated using the formula: SF = number of colonies/ number of cells seeded × PE. End points included the surviving fraction at each dose level. The linear-quadratic (LQ) model was calculated using the following equation: $SF = e^{-(\alpha D + \beta D^2)}$, where D is the dose in Gy, α is the cell kill per Gy of the initial linear component (on a log-linear plot) and β is the cell kill per Gy² of the quadratic component of the survival curve.

Cell Cycle Analysis

MCF7, HCC1954 or SUM159 cells received 0 or 5 Gy irradiation using the X-RAD 320 irradiator (Precision X-ray) and harvested 24, 48 and 72 h postirradiation. These cells were fixed with 70% ethanol, stained with propidium iodide (PI, 0.5 mg/ml, cat. no. 81845; Sigma-Aldrich) in IFA buffer (10 mM HEPES, 150 mM NaCl, 4% FBS and 0.1% Na₃N containing RNase (10 mg/ml, Sigma-Aldrich), then analyzed by flow cytometry (FACSCanto™, BD Biosciences, San Jose, CA). Analysis of data was performed with FCS Express (De Novo™ Software, Los Angeles, CA) or FlowJo® (Tree Star Inc., Ashland, OR).

Western Blot Analysis

Cell or tumor extracts were prepared in lysis buffer (containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 2 mM DTT, 1% NP40, 10 mM β -glycerophosphate and 1 mM Na₃VO₄) supplemented with 1× protease inhibitor cocktail (cat. no. P8340; Sigma-Aldrich). Protein concentration was measured using protein assay dye reagent (cat. nos. 500-0113–500-0115; Bio-Rad® Laboratories Inc., Hercules, CA) according to the manufacturer's instructions. For Western blot analysis, protein lysates were mixed with equal volumes of 2× Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) containing 5% β -mercaptoethanol (cat. no. 161-0710), resolved by SDS-PAGE (cat. no. 456-0196) and transferred to a polyvinylidene difluoride membrane (cat. no. 162-0177) (all from Bio-Rad Laboratories), was blocked with either 5% nonfat milk or 3% BSA in TBS-T (cat. no. 46-012-CM; Corning® Inc., Corning, NY). The membrane was then probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibody and developed with ECL-detecting reagents (cat. no. 34078; Thermo Scientific™, Rockford, IL) and autoradiography film (cat. no. F-BX810; Phenix Research Products, Chandler, NC). The band intensity was quantified using ImageJ software (NIH, Bethesda, MD). The following primary antibodies were used: FAS, cleaved PARP (Asp214), p-Chk1 (133D30) and p-Chk2 (cat. nos. 8023, 9541, 2348S and 2661S, all from Cell Signaling Technology® Inc., Danvers, MA); FAS ligand (C-178) and DR5 (D-6) (cat. nos. sc-6237 and sc-166624, both from Santa Cruz Biotechnology® Inc., Dallas, TX), p53 (MS-187-PO; NeoMarkers, Fremont, CA), p21 (CP74, cat. no. MS891PO; Fisher Scientific) and p-ATM, ATM (kindly provided by Dr. Michael B. Kastan, Duke University). The band intensity was quantified using ImageJ software.

Orthotopic Tumor Model and Radiation Treatment

MCF7, HCC1954 or SUM159 cells (10^7 cells) were mixed with Matrigel® (cat. no. 356237; Corning Inc.). Nude mice were anesthetized and a small subcutaneous incision was made in the dorsal skin to expose the fat pad. The prepared cell/Matrigel solution (100 μ l) was injected into the fat pad. For MCF7 cells, mice received supplemental estrogen pellets (17 β -estradiol, 1.7 mg, 60-day release, cat. no. SE-121; Innovative Research of America, Sarasota, FL) subcutaneously three days before tumor implantation. Once tumors reached approximately 100–200 mm³, mice were exposed to different radiation doses (energy source: X-ray tube; energy: 320 kV; filtration: 2 mm aluminum; and dose rate: 3.464 roentgens/s) using the X-RAD 320 (Precision X-ray). Lead shielding jigs were used to block radiation to the rest of the body. Tumors were measured with calipers three times a week until tumor size reached 1,200 mm³. Tumor volume was calculated as $V = [(A^2) \times (B) 3 \pi]/6$, where A is the shortest tumor diameter and B is the longest. For the 50% tumor control dose (TCD₅₀) study, the X-RAD 225 Cx irradiator (energy source: X rays; energy: 225 kV; filter: 0.3 mm copper; and dose rate: 14.667 roentgens/s) (Precision X-ray) was used to deliver higher doses of radiation (10–50 Gy) with a special collimator (10 $\times$ 10 mm) to limit the dose to surrounding normal tissues. Commissioning was completed after initial installation of the tube for filtration, beam parameters and dosimetry. Checks for constancy are completed annually and after every machine repair.

FAS Modulation and Radiation Treatment

Control or FAS-silenced MCF7 cells were transplanted orthotopically into nude mice. When the tumor reached 100–200 mm³, mice were randomized and the tumor area received 0 or 10 Gy irradiation using the X-RAD 320 irradiator (320 kvp, 10 mA and F1 filter; Precision X-ray). Lead shielding jigs were used to block radiation to the rest of the body. For HCC1954 and SUM159 cells, tumor cells were transplanted into the dorsal mammary fat pad of nude mice. When the tumor reached 100–200 mm³, mice were randomized into four treatment groups. Mice were injected intratumorally with either control IgM or FAS agonistic antibody, CH11, 6 h prior to either 0 or 20 Gy irradiation. Tumor growth was evaluated three times a week until tumor size reached 1,200 mm³.

Tumor Growth Curve Modeling and Statistical Analysis

In vitro clonogenic survival—To compare radiation responses on clonogenic survival among three breast cancer cell lines, we fitted a model of the form [Eq. (1)],

$$\text{Log}(SF_c(d)) = \beta d + \beta_c d + \varepsilon_{cd}, \quad (1)$$

where $SF_c(d)$ was the surviving fraction for cell line c at dose d ($= 0, 2, 5$ or 8 Gy). The model approximated this in terms of a baseline slope β , which represented the rate of decrease for MCF7 and effects β_c for the other cell lines. Finally, $\beta_c d$ was the measurement error. The fitted model gave the steepest decrease for MCF7, at the rate of 0.97/ Gy. Rates of decrease for HCC1954 (0.54/Gy) and SUM159 (0.43/ Gy) were significantly slower ($P = 0.001$ and 0.0003 , respectively) than MCF7.

In vivo tumor growth delay—The effects of radiation on breast tumor growth were analyzed using a combined Cox regression model [Eq. (2)] (12),

$$\lambda(t, d, c) = \lambda(t, 0, MCF7) \exp(\alpha_c + \beta d + \beta_c d), \quad (2)$$

where c represented cell line MCF7, HCC1954 or SUM159. The baseline hazard represented the hazard for MCF7 with 0 Gy dose. The model explains variation in survival in terms of an effect of cell line SUM159 or HCC1954 (α_c) at 0 Gy dose and an effect of dose for MCF7 (β), as well as an additional effect of dose for SUM159 or HCC1954 (β_c). The outcome modeled was the time to exceed the volume end point (1.200 mm³) or the time to end of study (91 days).

TCD₅₀ study—For the TCD₅₀ study, a logistic model [Eq. (3)] (13) (<https://www.r-project.org/>) was used to estimate the dose-response curves for MCF7 and SUM159.

$$\log \frac{p_j(d)}{1 - p_j(d)} = \alpha + \alpha_{SUM} + \beta \log(d+1), \quad (3)$$

where $p_j(d)$ was the probability of tumor control in cell line j (MCF7 or SUM159) at dose $d = 0, 10\text{--}50$ Gy. The model explained this in terms of α , a baseline effect for MCF7, α_{SUM} , an additional effect for SUM159 and β represents the steepness of the dose-response curve.

FAS silencing and radiation response in MCF7—To compare tumor growth curves between control and FAS-shRNA-treated MCF7 cells, we fitted a combined Cox regression model of the form [Eq. (4)],

$$\lambda(t, d, c) = \lambda(t, 0, MCF7) \exp(\alpha_c + \beta d), \quad (4)$$

where c stood for cell line (MCF7 control or MCF7 FAS-silenced). The baseline hazard represented the hazard for MCF7 control (0 Gy) cells. The model explained variation in survival in terms of an effect of cell line with FAS-silenced, α_c , an effect (β) of dose d .

FAS activation and radiation response in SUM159 and HCC1954—To analyze tumor growth curves in SUM159 and HCC1954 cells treated with CH11 Ab and radiation, a Cox regression model [Eq. (5)] was used, where $\lambda_g(t)$ was the instantaneous hazard of exceeding the tumor volume threshold in group g (control, CH11 or CH11 after irradiation). The model approximates this hazard in terms the baseline hazard (radiation) and a group effect (hazard ratio) α_g .

$$\lambda_g(t) = \lambda_{RT}(t) \exp(\alpha_g), \quad (5)$$

RESULTS

Radiation Response in Human Breast Cancer Cells In Vitro

To study the sensitivity of specific breast cancer phenotypes to ionizing radiation, MCF7 (luminal), HCC1954 (HER2⁺) and SUM159 (basal) cells received single doses of 2, 5 or 8 Gy. A clonogenic assay was performed to evaluate cellular survival (Fig. 1A and Supplementary Table S1; <http://dx.doi.org/10.1667/RR14664.1.S1>). Consistent with clinical data suggesting that ER⁺ tumors derive a greater benefit from radiotherapy, our results indicated that among the breast cancer cells studied here, MCF7 were the most radiosensitive and SUM159 were the most radioresistant.

To understand the molecular mechanisms behind this differential response, these tumor cells received a 5 Gy dose and then analyzed for DNA content and cell cycle progression 24, 48 and 72 h postirradiation. Radiation induced both G₁ and G₂/M arrests in MCF7 cells, which express wild-type p53. In contrast, HCC1954 and SUM159 cells, which have mutant p53, underwent only G₂/M arrest in response to radiation (Fig. 1B). Radiation-induced cell cycle arrest was seen 48 h postirradiation in MCF7 and HCC1954 cells but all three cells had returned to baseline 72 h postirradiation.

ATM is the most proximal signal transducer induced by ionizing radiation in response to DNA damage, and subsequently activates cell cycle checkpoints. In our study, radiation induced ATM, Chk1 and Chk2 phosphorylation in all three cell lines, indicating the signaling pathways to sense DNA damage were activated in these cells (Fig. 1C). In addition, radiation-induced p53 and p21 accumulation was observed only in MCF7 cells and not in SUM159 and HCC1954 cells (Fig. 1C). Overall, these data suggested that radiation activates different cell cycle checkpoints in different breast cancer subtypes depending on their p53 status.

Radiation Response in Human Breast Cancer Cells In Vivo

We next investigated radiation response *in vivo* using the orthotopic breast cancer xenograft model. MCF7, HCC1954 and SUM159 cells were implanted orthotopically into the dorsal mammary fat pad of nude mice. Once the tumor volume reached 100–200 mm³, mice were randomized and received a single dose of 10, 15 or 20 Gy. We chose this large single-dose approach (as opposed to a more conventionally fractionated approach) given the trends in radiotherapy practice towards extreme hypofractionation, as well as our own ongoing institutional study in early-stage breast cancer; the initial *in vitro* hypothesis, tested *in vivo* in the current study, originated from this clinical trial utilizing a large single-dose preoperative approach.

Our results showed that radiation inhibited MCF7 and HCC1954 tumor growth in a dose-dependent manner, while radiation treatment had little effect on SUM159 tumor growth (Fig. 2A). Four out of 6 MCF7 tumors vanished after receiving a 20 Gy dose, but even a 10 or 15 Gy dose appeared to result in a growth delay relative to nonirradiated controls. For HCC1954 tumors, we started to see a growth delay at a 15 and 20 Gy dose. However, even after receiving a 20 Gy dose, these tumors regrew, therefore no mice were cured. In contrast, even after 20 Gy irradiation SUM159 tumors were resistant to radiation.

To determine the TCD₅₀ dose in the most sensitive and resistant phenotypes, SUM159 tumor-bearing mice were irradiated with higher doses of up to 50 Gy. A logistic dose-response model as described in Materials and Methods was used to estimate the dose-response curves for MCF7 and SUM159 cells. The estimated TCD₅₀ value for MCF7 was 19.3 Gy with a 95% confidence interval (CI) of (14.09, 24.51) Gy, while the estimated TCD₅₀ value for SUM159 was 44.9 Gy with a 95% CI of (38.28, 52.49) Gy. (Fig. 2B and Supplementary Table S2; <http://dx.doi.org/10.1667/RR14664.1.S1>). The estimated isoeffect value ($\alpha_{SUM} = -15.54 \pm 6.77$) between MCF7 and SUM159 indicated a significant difference in their dose-response curves ($P = 0.02$).

To compare radiation response and tumor growth in these breast cancer cells, a time-to-end point-based analysis and Kaplan-Meier survival curves were performed. A combined Cox regression model was used to model survival as a function of radiation dose (12) (Fig. 2C and Supplementary Table S3; <http://dx.doi.org/10.1667/RR14664.1.S1>). Compared to nonirradiated MCF7, both HCC1954 and SUM159 induced an increase in the hazard ratio of mortality. These data suggested inherently different growth rates among the three tumors, where MCF7 is slower growing and SUM159 and HCC1954 are aggressively growing tumors. As a function of dose, radiation significantly decreased the log hazard of mortality by 0.11/Gy in MCF7 tumor-bearing mice, suggesting that increasing radiation dose improved survival in MCF7 tumor-bearing mice. The median survival time increased from 34 days at 0 Gy to 44.5 days at 10 Gy. Median survival was not reached at 15 and 20 Gy. We also observed an increased survival in HCC1954 tumor-bearing mice from 17 days at 0 Gy to 26, 57 and 56 days at 10, 15 and 20 Gy respectively. By contrast, survival times for SUM159 tumor-bearing mice were much shorter overall (11–13 days) and with no apparent effect of radiation dose. The effects of radiation on HCC1954 tumor growth were not significantly different from MCF7. By contrast, SUM159 tumor growth appeared to be totally insensitive to radiation dose, and the difference in dose sensitivity relative to MCF7 was statistically significant.

Effects of Radiation on FAS and FAS ligand Expression in Human Breast Cancer Xenografts

FAS is a critical modulator of programmed cell death. Our previously published studies have shown that FAS expression was distinctly different before and after irradiation in both breast cancer patients and breast cancer cell lines. Modulation of FAS in breast cancer cell lines altered radiation response (10). In this study, we validated that FAS was induced in MCF7 tumors ($P = 0.058$) 24 h postirradiation but showed no change in SUM159 tumors. On the other hand, FAS ligand was not changed by radiation in either tumor (Fig. 3).

We also evaluated the correlation between FAS response to radiation and tumor growth delay. In MCF7 tumors collected 15–90 days after 0 or 10 Gy irradiation (insufficient tumor at 15 and 20 Gy), our data indicated that radiation induced FAS expression in MCF7 tumors, and there does appear to be a correlation between the extent of FAS induction and associated tumor growth delay (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14664.1.S1>). We also analyzed FAS expression in HCC1954 tumors collected 10–90 days postirradiation (Supplementary Fig. S2). As expected, HCC1954 tumors expressed high basal levels of FAS

with no significant change after irradiation. In this group, we again observed trends between FAS induction and tumor growth delay at 15 and 20 Gy irradiation. While an increased tumor sample size is needed to validate this correlation, overall these findings are consistent with our previous data, suggesting that FAS induction is associated with tumors having a more robust radiation response.

FAS Silencing and Radiation Response in Radiosensitive MCF7 Xenografts

To investigate whether FAS modulation can affect radiation response in radiosensitive MCF7 cells, we used shRNAs to silence FAS and achieved 90% FAS reduction (Fig. 4A). Control and FAS-silenced MCF7 cells were implanted orthotopically into nude mice. Once the tumor volume reached 100–200 mm³, mice were randomized and irradiated with 0 or 10 Gy. We found that a 10 Gy dose inhibited both control and FAS-silenced MCF7 tumor growth by reducing the hazard ratio of mortality at the rate of 0.27/Gy (Fig. 4B–C and Supplementary Table S4; <http://dx.doi.org/10.1667/RR14664.1.S1>). The radiation effects between the control and FAS-silenced MCF7 cells were similar ($P = 0.07$). We also considered an interaction between dose and cell line (not shown), but found nothing significant. Overall, these results suggested that silencing of FAS may not, in isolation, negatively affect radiation response in MCF7 tumors. However, with only 90% reduction of FAS, the residual FAS could still function to respond to radiation-induced cell death. In addition to FAS, death receptor 5 (DR5), another candidate gene from our previous gene analysis, was also induced after irradiation in MCF7 tumors (Fig. 4D) and may be sufficient to compensate for the reduction in FAS signaling.

FAS Activating Antibody in Radioresistant SUM159 and HCC1954 Xenografts

We have previously shown that HCC1954 and SUM159 tumors did not have high levels of change in gene expression, compared to MCF7 tumors (10). However, both tumors expressed high basal levels of FAS that were not affected by radiation. To determine whether the FAS signaling pathway was defective in these tumors, contributing to their radioresistance, we injected FAS activating antibody (Ab) CH11 intratumorally into SUM159 or HCC1954 tumor-bearing mice. We observed cleaved caspase 8 and cleaved poly (ADP-ribose) polymerase (PARP) in the tumors (Fig. 5), indicating activation of the apoptosis pathway by CH11 injection. We also found that the single dose of CH11 (6 µg) was more effective in inducing apoptosis than multiple doses (3 µg in two consecutive days). Overall, these data suggested that although FAS signaling was not activated in response to radiation, the pathway was intact and could be activated by CH11.

As such, we hypothesized that activating FAS signaling together with radiation may have a synergistic apoptotic effect in these cell lines to improve their radiation response. To test our hypothesis, SUM159 tumor-bearing mice were injected intratumorally with either control IgM or CH11 Ab (6 µg/mouse). Mice received a single 20 Gy dose 6 h after treatment. Of note, intratumoral injection of either control or CH11 Ab caused a small amount of tumor fluid leakage and tumor shrinkage in all treatment groups (Fig. 6A). Among the four treatment groups, animals treated with combined CH11 and radiation had the best survival (Fig. 6B and Supplementary Table S5; <http://dx.doi.org/10.1667/RR14664.1.S1>), although this did not reach statistical significance compared to radiation alone ($P = 0.47$).

In HCC1954 tumor xenografts, tumors were more sensitive to radiation compared to SUM159 cells. In the combined treatments group, two of the tumors vanished entirely, and this group had the best survival compared to the group treated with radiation alone (Fig. 6C–D and Supplementary Table S6), although again, this was not statistically significant ($P=0.23$).

DISCUSSION

Radiotherapy is an important contributor to both local control and overall survival in breast cancer patients. Currently, few distinctions are made, based on breast tumor biology, for dose, concurrent drug delivery or other radiotherapy modulations. In contrast, systemic therapy is almost entirely subtype-based, and there is compelling clinical data to suggest that radiotherapy response may be similarly distinct (9).

In our previously published studies, we identified an association between breast cancer subtype and gene expression profiles in response to radiation (10). Here, we expanded our study and investigated radiation response in a mouse model of breast cancer. Our data indicate that although biology certainly influences inherent growth rate, response to radiation also appears unique to breast tumor subtype *in vivo*. MCF7 cells (luminal) were both slow growing and radiosensitive. In contrast, SUM159 cells (basal) grew rapidly and were radioresistant.

To study the underlying mechanisms, we investigated radiation-induced cellular response in these breast cancer cell lines. ATM is a critical player in detecting DNA damage and activating cell cycle checkpoint pathways (14). After irradiation, ATM can phosphorylate and stabilize p53 through Chk1 or Chk2-mediated pathways. Accumulation of p53 then transcriptionally activates the cyclin-dependent kinase inhibitor p21, induces cell cycle arrest and ultimately, apoptosis (15–17). In this study, we found that ATM signaling was activated in all three breast cancer cell lines. However, downstream signaling and cell cycle arrest were unique for each cell type, depending on their p53 status.

It has been shown that p53 mutation or loss of function in tumor cells increases radiation resistance either by increasing DNA repair processes or enhancing cellular tolerance to DNA damage. In addition, certain types of p53 mutations, so-called gain-of-function mutants, can increase therapy resistance (18–21). Furthermore, radiation can induce other downstream targets, such as the breast cancer type I susceptibility protein (BRCA1), p53 binding protein 1 (53BP1) and mismatch repair proteins. The role of these downstream targets, as well as the specific effects of p53 itself on subtype-specific radiation response, will require additional investigation.

Nevertheless, one such downstream target, FAS, was identified in our previously published study by showing consistent and significant induction after irradiation in both ER⁺ breast cancer patients and breast cancer cell lines (10). Radiation has been shown to upregulate FAS in MCF7 cells with wild-type p53 (11). However, our previous data suggested that radiation-induced FAS expression may involve a p53-independent pathway (10). In this study, we have validated radiation-induced FAS expression in MCF7 tumors independent of

FAS ligand expression. On the other hand, SUM159 tumors expressed high basal levels of FAS and showed no change in FAS or FAS ligand after irradiation. This data, in conjunction with our prior work, suggest that tumors with FAS upregulation after irradiation are able to activate the programmed cell death pathway and have a more robust response to radiation. In contrast, radioresistant tumors do not appear to activate FAS after irradiation. However, downstream FAS signaling remains intact in these cells and can be stimulated by FAS activating antibody.

We also investigated the potential therapeutic effect of FAS modulation *in vivo*. Previously published studies have shown several approaches to target FAS/FAS ligand signaling for cancer therapy, including administration of FAS agonist antibodies or FAS ligand recombinant proteins (22–26), FAS ligand gene therapy (27–29) or use of chemotherapeutic drugs to induce FAS or FAS ligand expression (30–32). Here, to overcome radioresistance in SUM159 and HCC1954 tumors, we hypothesized that activating FAS signaling with radiation may have a synergistic apoptotic effect. Our data indicated that FAS activating Ab and radiation exposure delayed tumor growth and resulted in improved numerical, though not statistical, survival in both SUM159 and HCC1954 tumors. These data suggest that with further optimization, preactivation of the FAS signaling pathway may have the potential to enhance radiation-induced tumor death.

Nevertheless, there are limitations to our studies. The differential *in vivo* effect of radiation does appear to be consistent with clinical findings in thousands of breast cancer patients (9). However, we only utilized one cell line per breast cancer subtype to explore this question. With larger sample sizes, additional cell lines and where feasible, use of patient-derived xenograft models, we would likely be able to characterize the subtype-specific effect of radiation more fully. In addition, FAS modulation appears to have a modest effect on radiation response *in vivo*. Local injection may not provide optimal FAS activation, but systemic delivery has been associated with normal tissue toxicity. It may be that an alternative delivery system, such as a FAS aptamer, could enhance the radiosensitizing properties of FAS without increasing side effects; this is being investigated in our laboratory.

In conclusion, we have demonstrated that there is inherent variation in radiation response *in vivo* among breast cancer subtypes. Although FAS modulation was linked to radiation response *in vitro*, we did not observe a statistically significant impact of FAS activation in the *in vivo* setting. However, larger sample sizes representing the full biological spectrum of breast cancer are required to definitively characterize the role of FAS in radiation response. In our unique cohort of gene expression data from breast cancer patients treated with preoperative radiation, other candidate radiation response biomarkers associated with apoptosis, immune response and cell cycle control pathways have also been identified. Exploration of these genes in conjunction with or as an alternative to FAS in modulating subtype-specific radiation response is ongoing and will provide an essential foundation for breast radiotherapy tailored to tumor biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Radiation response in breast cancer cells *in vitro*. Panel A: Clonogenic survival in irradiated MCF7, HCC1954 and SUM159 cells. $*P < 0.05$. MCF7 cells were the most radiosensitive and SUM159 the most radioresistant. Panel B: Cell cycle analysis. Radiation-induced G₁ and G₂ arrest in MCF7 cells, but only G₂ arrest in HCC1954 and SUM159 cells. At 72 h postirradiation, the cells had returned to baseline. Panel C: ATM, Chk1, Chk2 activation and p53 and p21 expression by Western blot. Radiation activated ATM signaling in all three cell lines, but increased p53 and p21 expression only in p53 wild-type MCF7 cells.

FIG. 2.

Radiation response in breast tumor xenografts. Panel A: Effects of radiation on MCF7, HCC1954 and SUM159 tumor growth ($n = 5-6$). Data represent individual mice. One mouse in the HCC1954 nonirradiated (0 Gy) group with a tumor less than 100 mm³ at 90 days was omitted. Consistent with our *in vitro* results, MCF7 cell tumors were the most radiosensitive and SUM159 the most radioresistant. Panel B: Radiation dose-response curves for local tumor control at 90 days ($n = 4-6$). *TCD₅₀. Estimated TCD₅₀ for MCF7 was 19.3 and 44.9 Gy for SUM159. Panel C: Kaplan-Meier survival curves.

FIG. 3.

Effects of radiation on FAS and FAS ligand expression in breast tumor xenografts. FAS and FAS ligand expression in MCF7 (panel A) and SUM159 (panel B) tumors by Western blot. Data represent individual mice. The graph shows the ratio of the protein normalized to actin. Radiation induced FAS in MCF7 ($P = 0.058$, Wilcoxon rank sum test) but not in SUM159 tumors. FAS ligand was not changed by radiation in either cohort.

FIG. 4.

FAS silencing and radiation response in radiosensitive MCF7 xenografts. Panel A: FAS expression in control or FAS-silenced MCF7 cells by Western blot. Panel B: Effects of radiation on control or FAS-silenced MCF7 tumor growth (n = 4–5). Data represent individual mice. The effect of radiation was similar in both groups, suggesting that FAS silencing may not be sufficient, in isolation, to negatively affect radiation response. Panel C: Kaplan-Meier survival curve. Panel D: DR5 is increased in control MCF7 tumors and may

compensate for loss of FAS signaling. Data represent individual mice. The graph shows the ratio of the protein normalized to actin. * $P < 0.05$, Wilcoxon rank sum test.

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FIG. 5.

FAS signaling activation. SUM159 cells were implanted into the dorsal mammary fat pad of nude mice. Mice received intratumoral FAS activating CH11 Ab as a single dose of 0, 3 or 6 μg or 3 μg in two consecutive days. Tumors were collected 6 h after treatment for assessment of apoptosis signaling pathway activation by Western blotting. The FAS signaling pathway was intact and could be activated by FAS activating antibody CH11.

FIG. 6.

FAS activating antibody in radioresistant SUM159 and HCC1954 xenografts. Panel A: Effect of CH11, radiation and the combination on SUM159 (panels A and B) and HCC1954 (panels C and D) tumor growth (n = 5). Data represent individual mice. Combined CH11 and radiation treatments resulted in the highest survival in both lines, although this did not reach statistical significance compared to radiation alone.