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TGFβ1 Inhibition Increases the Radiosensitivity of Breast Cancer Cells *In Vitro* and Promotes Tumor Control by Radiation *In Vivo*

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Abstract

Purpose—To determine whether inhibition of TGFβ signaling prior to irradiation sensitizes human and murine cancer cells *in vitro* and *in vivo*.

Experimental Design—TGFβ-mediated growth and Smad phosphorylation of MCF7, Hs578T, MDA-MB-231, and T47D human breast cancer cell lines were examined and correlated with clonogenic survival following graded radiation doses with and without pretreatment with LY364947, a small molecule inhibitor of the TGFβ type I receptor kinase. The DNA damage response was assessed in irradiated MDA-MB-231 cells pretreated with LY364947 *in vitro* and LY2109761, a pharmacokinetically stable inhibitor of TGFβ signaling, *in vivo*. The *in vitro* response of a syngeneic murine tumor, 4T1, was tested using a TGFβ neutralizing antibody, 1D11, with single or fractionated radiation doses *in vivo*.

Results—Human breast cancer cell lines pretreated with TGFβ small molecule inhibitor were radio-sensitized, irrespective of sensitivity to TGFβ growth inhibition. Consistent with increased clonogenic cell death, radiation-induced phosphorylation of H2AX and p53 was significantly reduced in MDA-MB-231 triple-negative breast cancer cells when pretreated *in vitro* or *in vivo* with a TGFβ type I receptor kinase inhibitor. Moreover, TGFβ neutralizing antibodies increased radiation sensitivity, blocked γH2AX foci formation, and significantly increased tumor growth delay in 4T1 murine mammary tumors in response to single and fractionated radiation exposures.

Conclusion—These results show that TGFβ inhibition prior to radiation attenuated DNA damage responses, increased clonogenic cell death, and promoted tumor growth delay, and thus may be an effective adjunct in cancer radiotherapy.

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Introduction

Ionizing radiation is an effective cancer treatment modality that is administered in a manner that maximizes tumor damage while minimizing effects on normal adjacent tissue (1). Further benefit can be achieved through administration of drugs or biological agents that either promote tumor cytotoxicity or protect normal tissue from dose-limiting toxicity, such as fibrosis. TGF β is a candidate target whose inhibition could potentially do both.

There is substantial evidence that TGF β plays a crucial role in the response to ionizing radiation (2). TGF β is a pleio-tropic cytokine that is important in normal tissue homeostasis, regulates inflammation and immune responses, and suppresses epithelial proliferation. TGF β is activated in irradiated tissues, presumably because the latent TGF β complex has a specific redox-sensitive conformation activated by reactive oxygen species, which are generated by radiation (3). Some preclinical models suggest that radiation-induced TGF β contribute to metastasis (4); consistent with this irradiated cells are primed to undergo TGF β -mediated epithelial-mesenchymal transition that increases motility and invasion (5, 6). Radiation-induced TGF β activity can also be sustained beyond an acute response, which may drive function-compromising fibrosis, a common sequel following radiotherapy, in susceptible tissues (7–11). Significant experimental support for a critical role of TGF β in radiation-induced fibrosis is provided by studies in which blocking TGF β production or signaling significantly reduces fibrosis in preclinical rodent models (8, 9, 12–15). This has led to recognition that TGF β inhibition following radiotherapy could prevent normal tissue toxicity due to fibrosis (9, 11, 16), although this concept awaits testing in clinical trials.

A new role for TGF β in mediating the rapid execution of the DNA damage response (DDR) has been identified (reviewed in ref 2). Perhaps the first indication of its critical role was the observation that epithelial tissues of *Tgfb1* heterozygote embryos exposed *in utero* to 5 Gy showed significantly less radiation-induced apoptosis and *Tgfb1* null embryos fail to undergo either apoptosis or inhibition of cell cycle (17). TGF β depletion by gene knockout or transient depletion by TGF β neutralizing antibody also reduced radiation-induced p53 phosphorylation *in vivo* (17). Subsequent studies by Boothman and colleagues showed that TGF β initiates a radiation survival mechanism dependent on secretory clusterin (18). However, the failure of the proximal DDR is attributed to compromised ataxia telangiectasia mutated (ATM) protein auto-phosphorylation and kinase activity, which decreases phosphorylation of critical DNA damage transducers γ H2AX, Chk2, p53, and Rad17 and in turn, abrogates cell fate decisions (19). As a consequence, both *Tgfb1* null murine epithelial cells and human cells in which TGF β signaling is pharmaceutically blocked are more radiosensitive, as measured by clonogenic survival (19). Furthermore, Rodemann and colleagues showed that TGF β 1 antisense also compromises ATM kinase-dependent phosphorylations in irradiated A549 lung cancer cells (20). ATM is a nuclear sensor of DNA damage that initiates, recruits, and activates a complex program of checkpoints for cell cycle, apoptosis, and genomic integrity and its loss or inhibition enhances radiosensitivity (see reviews in refs. 21, 22). As a consequence, radiosensitivity, as measured by clonogenic survival, increases in both murine epithelial cells from *Tgfb1* null mice and in human epithelial cells in which TGF β signaling is pharmaceutically blocked (19).

Most solid cancers escape TGF β growth regulation and amplify TGF β production, which in turn suppresses immuno-surveillance and enhances invasion and metastasis (23), thus leading to a protumorigenic environment. TGF β also compromises responses to chemotherapy (reviewed in ref. 24), by mechanism that remains undefined. Taken together, increased levels of TGF β protein in cancer, TGF β activation by radiation, and TGF β regulation of ATM kinase activity and DDR, suggest that TGF β could protect cancer cells

from DNA damage, thus decreasing the efficacy of radiotherapy. A small molecule inhibitor of TGF β signaling has shown efficacy in combination with radiation and chemotherapy in human glioblastoma xenografts (25). However, breast cancer often evades the growth-inhibitory action of TGF β by selectively eliminating cytostatic gene responses due to attenuated C/EBP β transcriptional control (26). Although TGF β control of ATM is not dependent on cell-cycle status per se in normal cells (19), it is unknown whether TGF β inhibition can increase radiosensitivity when growth regulation is truncated in breast cancer cells. Thus, to assess the therapeutic potential of TGF β inhibition in radiotherapy for breast cancer, we determined the relationship between sensitivity to TGF β -mediated growth inhibition, molecular responses to radiation, and radiosensitivity in human and murine breast cancer cells. This study shows that TGF β inhibition increases radiosensitivity of breast cancer cells irrespective of growth sensitivity, compromises DDR, and promotes radiation-induced tumor growth delay.

Materials and Methods

Cell culture

Human breast cancer cell lines MCF10A MCF7, MDA-MB-231, T47D and Hs578T cells, and murine breast cancer cell line 4T1 were obtained from American Type Culture Collection and were cultured in MEGM, 10% FBS-MEME, 10% FBS-DMEM, 10% FBS-RPMI, 10% FBS-DMEM, and 10% FBS-DMEM media, respectively, at 37°C with 5% CO₂. Cells were treated in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc.) containing either 500 pg/mL TGF β (R&D Systems), 400 nmol/L small molecule inhibitor of the TGF β type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole); Lilly designation HTS466284; Catalogue no. 616451, Calbiochem) or 1D11, a pan-isoform, neutralizing TGF β monoclonal antibody or 13C4, murine monoclonal isotype control antibody (Genzyme). For growth studies, cells were trypsinized and counted using a Coulter counter at 24-hour and 48-hour posttreatment. Cells were grown in complete media for 48 hours, followed by LY364947 treatment (400 nmol/L) in 10% SRM for 24 hours prior to irradiation with 2 Gy for γ H2AX foci induction by immunofluorescence and 5 Gy for DDR using immunoblotting.

Colony assay

Human breast cancer cells grown for 48 hours to 70% confluence were treated with 400 nmol/L of LY364947 kinase inhibitor for 48 hours before and 3-hour postradiation exposure with differential doses using a 250-kVp X-ray (0.61 Gy/min). Murine mammary 4T1 tumor cells were treated with 1D11 or 13C4 for 24 hours before irradiation to the indicated dose with Clinac 2300 C/D linear accelerator. Cells were trypsinized 3-hour postirradiation and were plated in triplicates at 3 dilutions into 6-well cell culture plates in serum containing media. Cells were allowed to grow for 10 to 12 days followed by fixing and staining with crystal violet. Colonies containing more than 50 cells were counted. To determine percent survival, colony forming efficiency was determined, averaged, and normalized to those of the nonirradiated control. For each radiation dose, the mean number of colonies obtained from 3 wells were corrected according to plating efficiency and used to calculate the cell survival at each dose (27). The significance of the difference between the dose responses was calculated by conducting a 1-way ANOVA test. We chose the hierarchical ANOVA models to control the heterogeneity of the data caused by the interaction of the 2 variables and to infer the statistical significant differences in the clonogenic survival between treatments for the given radiation doses using factorial ANOVAs followed by least significant difference 2-by-2 comparisons.

Immunoblot analysis

To examine molecular responses to TGF β or radiation, 7×10^5 cells were grown in complete media for 48 hour, followed by LY364947 treatment (400 nmol/L) in 10%SRM for 24 hours followed by exposure to 5 Gy, which were lysed after 1 hour, or treated with 500 pg/mL TGF β , which were lysed after 30 minutes. The extracts were subjected to immunoblot analysis with one of the following primary antibodies: Smad2 serine 465/467 phosphorylation at 1:500 (clone 138D4, Catalogue no. 3108, Cell Signaling), Smad2/ 3 at 1:500 (Catalogue no. 610842, BD Transduction Laboratories), p53 serine 15 phosphorylation at 1:500 (Catalogue no. 92845, Cell Signaling), p53 serine 20 phosphorylation at 1:500 (Catalogue no. 92879, Cell Signaling), and p53 at 1:500 (Clone DO-7 + BP53-12, Catalogue no. MS-738-P0 Neomarkers). Protein estimation was carried out using the BCA protein assay kit from Pierce. One hundred micrograms of protein was electrophoresed on a 4% to 15% gradient gel from BioRad and transblotted on polyvinylidene difluoride membrane. The membrane was incubated in blocking buffer and probed with one of the primary antibodies, washed 3 times for 10 minutes with 0.1% TBST, followed by incubation with secondary antibodies (goat anti-mouse, Catalogue no. 926-32220 and goat anti-rabbit, Catalogue no. 926-32211, Odyssey) for 1 hour at room temperature. The membrane was washed 3 times for 10 minutes with TBST 0.1% and scanned on the Odyssey LICOR system.

γ H2AX foci

Tumor cryosections or cells grown on chamber slides were fixed using 2% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 100% methanol for 20 minutes at -20°C . Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 hour, and incubated with a mouse monoclonal antibody against γ H2AX (clone JBW301, Upstate Biotechnology) overnight at 4°C followed by washes and incubation with Alexa-486 labeled anti-mouse secondary antibodies (Molecular Probes) for 1 hour at room temperature. Specimens were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and washed in PBS-Tween20 0.1% before mounting with Vectashield mounting medium (Vector Labs). Specimens were imaged using a 40 $\times$ objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epi-fluorescence. All images were acquired with a CCD Hamamatsu Photonics monochrome camera at $1,392 \times 1,040$ pixel size, 12 bits per pixel depth using the Metamorph imaging platform (Molecular Devices, Inc.).

In vivo tumor studies

All animal studies were conducted using protocols that had undergone appropriate review and approval at each institution. Severe combined immunodeficient mice/beige mice were injected subcutaneously at the UCSF Preclinical Therapeutics Core with 2×10^6 MDA-MB-231 breast cancer cells on each flank at the UCSF Preclinical Therapeutics Core. LY2109761 28 or vehicle control was administered once by oral gavage at day 29 when tumors were approximately 0.8 cm in diameter. Two hours later, the mice were irradiated or mock irradiated using a medial exposure body shield with 3 Gy Cs-radiation source. Tumors ($n = 3$ per treatment) were excised 1 hour after radiation exposure and frozen in OCT using dry ice/ethanol bath.

Balb/C mice were injected subcutaneously with 5×10^4 4T1 cells on left flank of the mice at NYU Medical Center and were treated at day 13, upon reaching approximately 100 mm³. Neutralizing antibody 1D11 or isotype control, 13C4 (29), were administered by intraperitoneally (i.p.) injection (5 or 50 mg/kg, kindly provided by Genzyme) 24 hours before tumors were locally irradiated with a single dose of 8 Gy or 3 doses of 12 Gy on 3 consecutive days. Briefly, mice were lightly anesthetized by i.p. injection of Avertin (240

mg/kg) and then positioned on a dedicated plexiglass tray to irradiate a field including the tumor with 5-mm margins using a Clinac 2300 C/D linear accelerator (Varian Medical Systems) fitted with a 25-mm radiosurgery conical collimator (BrainLAB AG). Superflab bolus (1.5 cm tissue equivalent material) was placed over the tumor, and a source-to-skin distance of 100 cm was set. Radiation was delivered at 600 cGy/min with 6 MV X-rays. Mice were monitored thrice weekly for signs of toxicity and tumors volumes were measured with a caliper. Tumors volumes were calculated as $\text{length} \times \text{width}^2 \times 0.52$.

Statistical analysis

Random coefficients regression was used to assess the effect of treatment on log tumor growth; log volumes were used in place of observed volumes because the change in log volume over the course of follow-up was well approximated as linear. The model to predict log tumor volume included elapsed time from baseline (defined as first day of measurement) as a numeric factor, treatment as a classification factor and the term representing the interaction of treatment with time. The interaction term was partitioned to derive tests that compared treatment arms in terms of tumor growth rate. The correlation structure imparted by the inclusion of multiple-dependent variable observations per animal was modeled by assuming observations to be correlated only when acquired from the same animal with the strength of correlation between observations inversely dependent on the elapsed time between observations (i.e., measures are more strongly correlated when taken closer together in time).

After conducting a Shapiro–Wilks test to verify the Gaussian distribution of the data, an ANOVA was used to compare treatment arms in terms of the normalized tumor volumes or tumor weight observed at each day of measurement. The error variance was allowed to differ across treatment arms to avoid the unnecessary assumption of variance homogeneity. All reported *P* values are 2-sided and were declared significant at the 5% level. All computations were carried out using commercially available software (SAS 9.0; SAS Institute).

Results

Human breast cancer cell responses to TGFβ

TGFβ regulation of ATM kinase activity and downstream phosphorylation targets does not depend on cycling status in nonmalignant MCF10A cells (19), but these cells are TGFβ responsive, whereas most breast cancers proliferate in the presence of active TGFβ signaling (30). Thus, we first established the relative TGFβ growth inhibition of MCF7, MDA-MB-231, Hs578T, and T47D breast cancer cell lines in comparison with MCF10A as a positive control (Fig. 1A). Among the breast cancer cell lines, only Hs578T cells were growth inhibited by TGFβ, similar to that observed in nonmalignant MCF10A cells. MCF7 and MDA-MB-231 cells were refractory to TGFβ-mediated growth regulation while T47D cells were slightly stimulated by addition of TGFβ.

Because breast cancer cell lines can selectively evade TGFβ growth regulation while maintaining signaling (26), we next examined activation of TGFβ canonical pathway as evidenced by Smad2 phosphorylation. Extracts from cells treated for 30 minutes with TGFβ showed a 2 to 12-fold increase in Smad2 phosphorylation, which was inhibited by pretreatment with LY364947, inhibitor of TGFβ receptor I (Fig. 1B). Thus all breast cancer cell lines, irrespective of their sensitivity to TGFβ-mediated growth inhibition, showed type I receptor kinase response to TGFβ treatment.

Inhibition of TGF β signaling increases radiosensitivity

We then determined clonogenic survival as a measure of the radiation sensitivity of these human breast cancer cell lines and evaluated the effect of TGF β signaling inhibition prior to irradiation. As previously reported (19), the radiation sensitivity of the TGF β responsive, nonmalignant cell line MCF10A was significantly increased when subjected to inhibition of TGF β signaling by TGF β type I receptor kinase small molecule inhibitor LY364947 (Fig. 2). Moreover, the radiation sensitivity of TGF β refractory cancer cell lines, MCF7 and T47D, and TGF β growth sensitive cancer cell line, Hs578T, was increased following TGF β inhibition (Fig. 2). The dose enhancement ratios at 10% survival (Table 1) varied between cell lines. Although representing a small sample of breast cancer cell lines, we found no apparent association between cancer cell subtype, estrogen receptor positivity or p53 mutation status, TGF β growth sensitivity, and the effect of LY364947 pretreatment on radiosensitization.

Inhibition of TGF β signaling attenuates DDR in MDA-MB-231 cells *in vitro* and *in vivo*

The MDA-MB-231 breast cancer cell line is designated triple negative and characterized as a basal subtype (31, 32). As in the other 3 breast cancer cell lines, LY364947 treatment prior to irradiation strongly radiosensitized MDA-MB-231 cells (Fig. 3A). Prior studies showed that compromised DDR exhibited by *Tgfb1* null mouse epithelial cells or following inhibition of TGF β signaling in nonmalignant human cells is due to significantly reduced ATM kinase activity (17, 19). ATM directly phosphorylates p53 at serine 15, and indirectly phosphorylates p53 at serine 20 (33). Consistent with increased radiosensitivity, p53 phosphorylation at both serine 15 and 20 was reduced in irradiated MDA-MB-231 cells treated with inhibitor (Fig. 3B). ATM also phosphorylates histone H2AX at serine 139, which is called γ H2AX, detected as discrete foci at surrounding DNA double-strand breaks caused by ionizing radiation (34). The formation of γ H2AX foci is widely used to monitor radiation-induced DNA breaks and to assay DNA rejoining defects (35). Consistent with increased radiosensitivity, p53 phosphorylation at both serine 15 and 20 was reduced in irradiated MDA-MB-231 cells (Fig. 3B). As found in our prior studies, radiation-induced γ H2AX foci formation was markedly reduced by pretreatment with LY364947 (Fig. 3C).

To determine whether TGF β inhibition could similarly affect the DDR *in vivo*, MDA-MB-231 cells were injected in immunocompromised mice to establish xenograft tumors. LY2109761 is a small molecule inhibitor that has similar activity as LY364947 (36), but is more pharmacokinetically stable, is orally available, and is the most potent *in vivo* inhibitor from this family of TGF β type I receptor inhibitors (37). Mice-bearing MDA-MB-231 tumors were treated with LY2109761 for 2 hours before exposure to 3 Gy. As found *in vitro*, radiation-induced γ H2AX foci were decreased in irradiated tumors treated with TGF β inhibitor, LY2109761 compared with vehicle-treated tumors (Fig. 3D). Smad2 phosphorylation was slightly increased by radiation and both constitutive and radiation-induced Smad2 phosphorylation was decreased by LY2109761 (data not shown).

TGF β neutralization in combination with radiation increase tumor growth delay

There are 3 pharmacologic routes to blocking TGF β ; neutralizing the ligand, inhibiting expression, or blocking signaling (36). The pharmacokinetic properties of antibody and small molecule kinase inhibitors result in considerable differences in the duration of TGF β signal modulation. At this point, the half-life of the TGF β type I receptor kinase small molecule inhibitors may not be optimal for use in the context of fractionated radiotherapy. Alternatively, several TGF β neutralizing antibodies are in clinical development that have shown safety and efficacy in fibrotic disorders (36, 38). We compared the efficacy of TGF β ligand capture using 1D11 pan-TGF β neutralizing antibodies to LY364947 *in vitro* using murine 4T1 breast tumor cells. Radiosensitization following 1D11 pretreatment (Fig. 4C)

was comparable with that following LY364947 pretreatment (Fig. 4A), which indicated that TGF β ligand sequestration and inhibition of the type I receptor kinase are functionally similar. As expected, both neutralizing antibody and small molecule inhibited Smad 2 phosphorylation activation in response to TGF β (Fig. 4B and D). Moreover, γ H2AX foci were significantly reduced in irradiated tumors from mice treated with 1D11 antibodies compared with those treated with 13C4 control antibodies (Fig. 4E).

TGF β is a key contributor to the immunosuppressive tumor environment (39); radiation-induced TGF β may hinder the development of anti-tumor immune responses that can be elicited by radiotherapy (40). Thus, to determine the effect of 1D11 antibody in conjunction with radiation treatment, we used 4T1 to establish preclinical breast tumors in immunocompetent mice. Mice-bearing 4T1 subcutaneous tumors were treated with TGF β neutralizing antibody, 1D11, or a control antibody, 13C4, at 5 mg/kg, i.p. Tumors were sham-irradiated or irradiated with a 8 Gy single dose 24 hours later. Some tumors were randomly selected for harvest 1 hour after irradiation to assess γ H2AX foci. As found with MDA-MB-231 cells treated with the small molecule inhibitor LY2109761, γ H2AX foci were reduced in irradiated tumor treated by pan-specific antibody against TGF β *in vivo* (Fig. 5A).

Although 4T1 tumor growth can be inhibited by TGF β blockade when treated 1 day posttumor inoculation due to an effect on tumor-promoting immune cells (41), the growth rate of established tumors was not affected by TGF β neutralizing antibody administered at this dose. As expected, irradiated tumors showed a significant tumor growth delay compared with unirradiated tumors (Fig. 5B and C). Tumor growth delay was further increased in mice that were both irradiated and treated with 1D11 neutralizing antibody compared with those that received radiation and 13C4 control antibody (Fig. 5D), with a significantly smaller average tumor volume ($P < 0.05$). No difference was observed with 1D11 treatment alone compared with no treatment controls ($P = 0.77$).

We speculated that increasing TGF β antibody (50 mg/kg) in the context of multiple radiation fractions (3×12 Gy) could possibly provide greater benefit (Fig. 6A). Even at this high dose, a single administration of 1D11 alone in established tumors did not significantly reduce 4T1 tumor growth ($P = 0.16$). Tumor growth rate was profoundly inhibited by treatment with multiple radiation fractions and further reduced by addition of 1D11 (Fig. 6B) compared with 13C4 ($P < 0.05$). Tumor weight at experiment termination (day 30) was also significantly reduced ($P < 0.05$) by combination treatment (Fig. 6C). No detectable toxicity, as evidenced by mouse husbandry or weight, was noted in mice treated with 1D11 antibodies for either dose (5 or 50 mg/kg) alone, or in combination with localized tumor irradiation (data not shown).

Discussion

We report herein that TGF β inhibition increased radiation sensitivity measured by clonogenic assay of breast cancer cells *in vitro* and tumor growth delay *in vivo*. As previously shown for chemotherapy (24), inhibiting TGF β promotes tumor response to radiation therapy. Our prior research using mouse and human epithelial cells showed that TGF β inhibition compromises radiation-induced ATM kinase activity and downstream effectors of the DDR, resulting in increased cell killing measured by clonogenic assay (19). All 4 human breast cancer cells and a murine tumor cell line were radiosensitized independent of sensitivity to TGF β -mediated growth inhibition, suggesting that this strategy would be effective across breast cancer subtypes.

Consistent with our earlier studies indicating diminished DDR following radiation exposure, TGF β inhibition prior to irradiation also resulted in reduced phosphorylation of H2AX and p53 in cultured triple-negative MDA-MB-231 breast cancer cells. Either human MDA-MB-231 xenografts or murine 4T1 tumors in mice treated with TGF β inhibitors prior to radiation exposure *in vivo* exhibited less γ H2AX foci formation, a nuclear marker of the rapid molecular radiation response. Moreover, 1D11 neutralizing antibodies enhanced the tumor growth delay after a single radiation exposure, which is consistent with a direct effect on radiosensitivity due to compromised DNA damage recognition. TGF β inhibition before radiation treatment was most effective in combination with fractionated radiation therapy. Notably, 1D11 antibody did not by itself affect tumor growth rate when administered to established tumors. It is quite conceivable that effects on angiogenesis (37) and immunity (42) could also contribute to greater efficacy of TGF β inhibition in combination with a fractionated course of radiation.

Teicher and colleagues showed that tumors secreting high levels of TGF β are more resistant to chemotherapies such as cis-platinum (43, 44). Cis-platinum treatment of MDA-MB-231 breast cancer cells increased both TGF β mRNA levels and the secretion of active TGF β , leading to growth arrest and repair of damage: as a result cells became more resistant to cis-platinum killing (43). Anti-TGF β antibodies enhanced cis-platinum induced DNA fragmentation in MDA-MB-231 cells, restoring cellular sensitivity to cis-platinum (44). Similarly, treatment of animals bearing cis-platinum-resistant tumors with TGF β neutralizing antibody or with the TGF β inhibitor, decorin, restores drug sensitivity of the tumor (43, 45, 46). The molecular mechanism(s) underlying this phenomenon remain to be elucidated; in our studies, TGF β signaling needs to be inhibited before DNA lesions occur to induce radiosensitivity (19). Inhibition of TGF β type II receptor by halofuginone was shown by Cook and colleagues to enhance the radiosensitivity of diverse tumor cell lines (47).

Exposure to either whole body or localized radiation induces TGF β production and activation, which in turn modulate late tissue effects (reviewed in ref. 8, 11, 16). Circulating TGF β was doubled 2 weeks after 4T1 tumor irradiation (Bouquet and Barcellos-Hoff, unpublished data). This persistent elevation could have deleterious consequences. Arteaga and colleagues showed that a radiation-induced systemic surge of TGF β -promoted metastatic spread (4). In these studies, irradiating nontumor-bearing sites of MMTV/PyVmT transgenic mice increased circulating levels of TGF β , circulating tumor cells, and lung metastases; these effects were all abrogated by administration of a neutralizing TGF β antibody. Reiss and colleagues identified antiangiogenic effects as an additional benefit of TGF β neutralizing antibody mediated by control of VEGF (48). Additional effects of TGF β neutralization on antitumor immunity (49) and decreased cell cooperation that produces a “protumor environment” (50) could also contribute to benefit in cancer therapy.

Given the established protumorigenic role of TGF β , its involvement in the response of cancer cells to treatment, and evidence that radiation-induced TGF β expression and activation lasts a period ranging from weeks to months in irradiated normal tissue (8), the concept of enhanced therapeutic index of radiotherapy from TGF β inhibition is further supported by our *in vitro* and *in vivo* studies. Of particular interest is that either abrogation of TGF β signaling by type I receptor kinase small molecule inhibitors or by ligand capture using neutralizing antibodies increased the radiosensitivity of diverse cancer cell lines *in vitro* and impaired DDR *in vivo*, which is the objective in the development of other therapies targeted to the DDR pathway (19, 20). Both MDA-MB-231 and 4T1 are models of triple-negative breast cancer, which has a poor prognosis. Recent studies from Reiss and colleagues show that TGF β inhibition alone effectively reduce metastasis in a similar setting (37), which lends additional credence to the use of TGF β inhibition in cancer therapy despite its tumor-suppressor activity. Our demonstration that short-term TGF β neutralizing

antibody increased tumor growth delay after a single radiation exposure and compromised tumor growth rate after fractionated radiation treatment in a syngeneic preclinical model without evidence of associated toxicity provides an additional route to therapeutic benefit. Our data argue for harnessing TGF β inhibitors at the time of radiotherapy could translate protumor TGF β biology into clinical benefit.

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Translational Relevance

Radiation therapy is an important treatment modality for breast and other cancers. We have shown that ionizing radiation induces TGF β activation and that TGF β inhibition compromises canonical radiation responses in epithelial cells via inhibition of ataxia telangiectasia mutated kinase activity. We hypothesized that inhibiting TGF β during radiotherapy could increase tumor response to radiation therapy if cancer cells maintain TGF β regulation of the DNA damage response. We show that human and mouse breast cancer cell lines were radio-sensitized when pretreated with either a small molecule inhibitor of the TGF β type I receptor kinase or pan-specific TGF β neutralizing antibody. Consistent with this, phosphorylation of γ H2AX and other DNA damage responses were significantly reduced by TGF β inhibition prior to *in vitro* or *in vivo* irradiation. Moreover, tumor growth delay following radiation was significantly greater when TGF β neutralizing antibodies were administered before either single or multiple radiation fractions. These preclinical studies suggest that TGF β inhibition during radiotherapy could provide significant benefit.

Figure 1.

TGFβ growth regulation and signaling in breast epithelial cell lines. A, the bar graphs show the growth response at 24 hours or 48 hours of TGFβ treatment in 5 breast epithelial cell lines. The untreated controls are designated as “UT” and “TGFβ” refers to TGFβ-treated cells. MCF10A cells show 14% ($P=0.38$) and 35% ($P=0.02$) growth inhibition at 24 hours and 48 hours of TGFβ treatment. Hs578T were growth inhibited by 9% ($P=0.36$) at 24 hours and 22% ($P=0.004$) at 48 hours of TGFβ treatment. Proliferation of MDA-MB-231 and MCF7 cells was unaffected by TGFβ whereas proliferation of T47D cells increased slightly. B, immunoblots of phospho-Smad2, total Smad2/3 and Actin from MCF7, T47D, Hs578t, MCF10A, and MDA-MB-231 cells treated with LY364947 for 48 hours followed by TGFβ for 30 minutes or sham treated. TGFβ treatment induced phosphorylation of Smad2, which was blocked by LY364947 pretreatment. These data indicate that TGFβ signaling through the type I receptor kinase is functional and LY364947 is effective in blocking the canonical pathway of TGFβ through TGFβRI. Quantifications of the ratios of phosphorylated protein/total protein normalized to untreated control are indicated below each lane.

Figure 2.

TGF β inhibition by small molecule inhibitor LY364947 radiosensitizes epithelial breast cell lines. Cells treated for 48 hours with 400 nmol/L of LY364947 prior to exposure to graded doses of radiation were plated 3-hour postirradiation for clonogenic survival assay. Closed circles (●) represent untreated irradiated controls whereas open circles (○) represent the colony forming efficiency of LY364947 treated, irradiated cells. Mean $\pm$ SD values of triplicate determinations are shown. LY364947 treatment significantly increases radiation sensitivity (ANOVA Tukey test): MCF10A, $P = 0.013$, Hs578T, $P = 0.005$, T47D, $P = 0.01$, and MCF7, $P = 0.01$.

Figure 3.

TGF β inhibition by LY364947 small molecule inhibitor radiosensitizes and attenuates DDR in MDA-MB-231 breast cancer cells *in vitro*. A, MDA-MB-231 cells treated for 48 hours with 400 nmol/L of LY364947 prior to exposure to graded doses of radiation were plated 3-hour postirradiation for clonogenic survival assay. Closed circles (●) represent untreated irradiated controls whereas open circles (○) represent the colony forming efficiency of LY364947 treated, irradiated cells. Mean $\pm$ SD values of triplicate determinations are shown for an assay representative of 3 experiments. LY364947 treatment significantly increases radiation sensitivity (ANOVA Tukey test, $P = 0.023$). B, MDA-MB-231 cells treated with LY364947 for 24 hours prior to irradiation with 5 Gy were subjected to immunoblot analysis to examine the effect of inhibiting TGF β signaling on p53 phosphorylated at serine

15 and at serine 20. TGF β inhibition prior to irradiation reduces p53 phosphorylation at both sites. The ratio of phosphorylated protein/total protein relative to untreated controls is indicated below each. C, induction of γ H2AX foci in cultured MDA-MB-231 cells irradiated with 2 Gy was decreased by pretreatment with LY364947. Cells exhibit nuclear immunostaining of γ H2AXfoci (green); nuclei are counterstained with DAPI (blue). D, γ H2AXfoci (green) were reduced in MDA-MB-231 xenograft tumors from mice-treated LY2109761 (bottom) and irradiated with 3 Gy (right). Nuclei are counterstained with DAPI (blue).

Figure 4.

TGF β neutralizing antibody 1D11 radiosensitized murine mammary cancer cells *in vitro*. A, colony forming efficiency of cells treated for 48 hours with LY364947, prior to irradiation. Closed circles (●) represent control cells whereas open circles (○) represent the colony forming efficiency of LY364947 treated irradiated cells irradiated to the indicated dose. Radiation sensitivity was increased by LY364947 treatment ($P = 0.024$, ANOVA Tukey test). A representative experiment of 3 is shown. B, immunoblots of phospho-Smad2 and total Smad2/3 from 4T1 cells treated with LY364947 for 24 hours followed by TGF β for 30 minutes or sham treated. The ratio of phosphorylated protein/total protein normalized to control is indicated below each. C, colony forming efficiency of cells treated for 24 hours

with 1D11 or 13C4 antibody control, prior to irradiation. Closed circles (●) represent control antibody-treated cells whereas open circles (○) represent the colony forming efficiency of 1D11 TGFβ neutralizing antibody-treated irradiated cells irradiated to the indicated dose. Radiation sensitivity was increased by 1D11 treatment ($P = 0.03$, ANOVA Tukey test). A representative experiment of 3 is shown. D, immunoblots of phospho-Smad2 and total Smad2/3 from 4T1 cells treated with 1D11 for 24 hours followed by TGFβ for 30 minutes or sham treated. The ratio phosphorylated protein/total protein normalized to control is indicated below each. E, 4T1 cells treated with LY364947 for 48 hours prior to irradiation with 2 Gy were immunostained for γH2AX (green) and DAPI (blue). Radiation-induced γH2AX foci were decreased by LY364947/TGFβ inhibition.

Figure 5.

TGF β neutralizing antibody 1D11 increases radiation-induced tumor delay growth *in vivo*. A, 4T1 tumors treated i.p. with 5 mg/kg 1D11 or 13C4 control antibody and irradiated at 8 Gy and harvested 1 hour later were immunostained for γ H2AX (green) and DAPI (blue). γ H2AX foci were decreased in tumors from mice treated with 1D11 (bottom) compared with the tumor treated with 13C4 (top). Low (left) and high (right) magnifications are shown. B, 4T1 tumor volumes from mice treated as above on day 13 with 13C4 control antibody (circles) or 1D11 TGF β neutralizing antibody (squares) with (open symbols) and without (closed symbols) radiation (8 Gy) at day 14. 1D11 treatment increased the response to radiation ($P = 0.01$) but did not itself affect tumor growth rate ($P = 0.77$). C, individual

tumor growth curves according to treatment. D, tumor growth delay compared with control was significantly increased by radiation and combination at day 23. n = 13 mice/group; *, <0.05; **, <0.01; ***, <0.001; NS, not significant.

Figure 6.

TGF β neutralizing antibody 1D11 treatment increases the response to multiple fractions of radiation *in vivo*. A, 4T1 tumor volume growth curves from mice treated i.p. on day 13 with 50 mg/kg 13C4 control antibody (circles) or 1D11 TGF β neutralizing antibody (squares). Tumors were treated with 3 fractions of 12 Gy (open symbols) initiated at day 14 for 3 consecutive days. 1D11 and radiation decreased tumor growth rate compared with radiation and 13C4 ($P < 0.05$). 1D11 treatment alone did not significantly affect tumor growth rate ($P = 0.16$). B, individual tumor growth curves according to treatment. C, tumor weight at experiment termination was significantly decreased by the treatment combination. $n = 5$ mice/group; *, <0.05 ; **, <0.01 ; ***, <0.001 ; NS, not significant.

Table 1

Characteristics of breast cancer cell lines and radiation sensitization

Cell line	Characteristics		TGFβ-mediated growth inhibition at 48 h	Dose enhancement ratio at 10% survival
	Subtype	ER ^a	P53 ^b	
MCF10A	Basal B	Neg	WT	1.18
Hs578T	Basal B	Neg	MT	1.04 ^c
MDA-MB-231	Basal B	Neg	MT	1.26
MCF7	Luminal	Pos	WT	1.7
T47D	Luminal	Pos	WT	1.26

^aER: Estrogen receptor status.

^bWT: Wild type; MT: Mutant.

^cDER was estimated based on extrapolation to 10% survival.