Resistance of Glioblastoma-Initiating Cells to Radiation Mediated by the Tumor Microenvironment Can Be Abolished by Inhibiting Transforming Growth Factor-β

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Abstract

The poor prognosis of glioblastoma (GBM) routinely treated with ionizing radiation (IR) has been attributed to the relative radioresistance of glioma-initiating cells (GIC). Other studies indicate that although GIC are sensitive, the response is mediated by undefined factors in the microenvironment. GBM produce abundant transforming growth factor-β (TGF-β), a pleotropic cytokine that promotes effective DNA damage response. Consistent with this, radiation sensitivity, as measured by clonogenic assay of cultured murine (GL261) and human (U251, U87MG) glioma cell lines, increased by approximately 25% when treated with LY364947, a small-molecule inhibitor of TGF-β type I receptor kinase, before irradiation. Mice bearing GL261 flank tumors treated with 1D11, a pan-isoform TGF-β neutralizing antibody, exhibited significantly increased tumor growth delay following IR. GL261 neurosphere cultures were used to evaluate GIC. LY364947 had no effect on the primary or secondary neurosphere-forming capacity. IR decreased primary neurosphere formation by 28%, but did not reduce secondary neurosphere formation. In contrast, LY364947 treatment before IR decreased primary neurosphere formation by 75% and secondary neurosphere formation by 68%. Notably, GL261 neurospheres produced 3.7-fold more TGF-β per cell compared with conventional culture, suggesting that TGF-β production by GIC promotes effective DNA damage response and self-renewal, which creates microenvironment-mediated resistance. Consistent with this, LY364947 treatment in irradiated GL261 neurosphere-derived cells decreased DNA damage responses, H2AX and p53 phosphorylation, and induction of self-renewal signals, Notch1 and CXCR4. These data motivate the use of TGF-β inhibitors with radiation to improve therapeutic response in patients with GBM. *Cancer Res; 72(16); 4119–29. ©2012 AACR.*

Introduction

Glioblastoma multiforme (GBM) remains a significant therapeutic challenge that poses an unmet medical need. Despite advances in radiation therapy and improvements in chemotherapeutics and targeted therapies, outcomes remain poor, with a median survival of 14.6 months when current standard of care, such as concurrent chemoradiation therapy and adjuvant chemotherapy, are used (1). Although the exact origin of GBM (and other malignant brain tumors) is unknown, it is hypothesized that a fraction of tumor cells have cancer stem cell characteristics (glioma-initiating cells, GIC) and true tumorigenic potential (2, 3). Originally proposed 25 years ago (4), it is generally hypothesized that the resistance of GIC contributes to the poor response to radiation and chemotherapy and inevitable tumor recurrence (reviewed in ref. 5). The exact mechanism of treatment resistance is unknown; however, GIC's intrinsic hyperactivation of the PI3K/Akt and PTEN pathways (6, 7) and increased activation of DNA damage checkpoint pathways (8) are expected to contribute.

The microenvironment, in addition, can contribute to radiation responses (reviewed in ref. 9). Tofilon and colleagues showed that GBM cells irradiated under orthotopic conditions have a greater capacity to repair DNA double-strand breaks than GBM cells irradiated *in vitro* (10). A critical component of the GBM microenvironment is the pleotropic cytokine transforming growth factor-β (TGF-β). TGF-β has a range of effects on the glioma microenvironment, including extracellular matrix deposition, angiogenesis, and invasion (reviewed in ref. 11). Both TGF-β1 and TGF-β2 have been implicated in autocrine tumor growth regulation (12). TGF-β2 is overexpressed in gliomas (13). Higher levels of TGF-β1 have been found in anaplastic gliomas (WHO grade III) than in GBM (WHO grade IV), indicating a potential role of TGF-β1 in the early stages of tumorigenesis (14).

The TGF-β family has been shown to play a role in both pluripotent stem cells (reviewed in ref. 15) and neural stem cells specifically (16). In addition, TGF-β has been implicated in GIC biology. Penuelas et al. showed that exposure of

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patient-derived tumor neurospheres to TGF-β increased the number of neurospheres in a dose-dependent fashion, and injection of these neurospheres into mice resulted in earlier appearance of more aggressive tumors (17). Ikushima and colleagues reported that autocrine TGF-β contributes to the tumorigenicity of the GIC population by activation of Sox4 and Sox2 (18). More recently, Seone and colleagues showed that TGF-β inhibitors affect a $CD44^{high}/Id1^{high}$ GIC population via Id1 and Id3, which they propose controls the "master regulators" of the TGF-β–GIC gene program, including leukemia inhibitory factor (LIF), Sox2, Sox4, and CD44 (19).

Ionizing radiation (IR) induces TGF-β *in vitro* and *in vivo* in both normal and cancer cells (20–22). We have shown previously that reactive oxygen species are possibly involved in the radiation-induced activation of TGF-β (23) and the process is mediated by a conformational change in latency-associated peptide (LAP)–TGF-β complex, allowing the release of active TGF-β1 (24). Our studies and others have directly linked TGF-β to DNA damage responses and radiosensitivity (25, 26). Inhibiting TGF-β decreases radiation-induced phosphorylation of p53, chk2, H2AX, and rad17, all of which are substrates of ataxia telangectasia mutated (ATM), a kinase critical in the molecular response to IR-induced DNA double-strand breaks. ATM, a member of the phosphatidylinositol 3-kinase (PI3-kinase) family, is hypothesized to be a master controller of cell-cycle checkpoint signaling pathways that are required for cell response to DNA damage and for genome stability. Moreover, there is evidence using proteomic profiling that prolonged TGF-β treatment of cells can affect DNA damage repair such as Rad51 in a Smad-dependent manner (27). Notably, breast cancer cell lines treated with a small-molecule TGF-β type I receptor kinase inhibitor showed increased radiosensitivity as measured by clonogenic assay and decreased DNA damage responses to radiation, including nuclear foci of the histone variant H2AX, regardless of sensitivity to TGF-β growth control. A syngeneic model of triple-negative breast cancer showed increased tumor growth delay in response to single or fractionated radiation treatment with the addition of TGF-β neutralizing antibodies during radiotherapy (28).

The current study was aimed at determining the effects of TGF-β inhibition on radiation sensitivity of the GIC population. To assess the therapeutic potential of TGF-β inhibition during radiotherapy, we determined the relationship between sensitivity to TGF-β-mediated growth inhibition, GIC formation, molecular responses to radiation, and radiosensitivity in human and murine GBM *in vitro* and *in vivo*. We determined that neurosphere cultures, compared with bulk populations, produce more TGF-β, whose inhibition significantly compromises both DNA damage response and self-renewal of GIC.

Materials and Methods

Cell culture

The murine glioma, GL261 (obtained from the National Cancer Institute-Frederick Cancer Research Tumor Repository, Frederick, MD; authenticated in 2012 by Idexx Radil) and human glioma U251 (a generous gift of Dr. Kevin Camphausen; authenticated in 2010 by Idexx Radil) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with 10% FBS (Sigma-Aldrich) and 1% pyruvate (Gibco). Human glioma U87MG [obtained from the American Type Culture Collection (ATCC) in 2011] cells were cultured in Eagle's Minimum Essential Medium (Gibco) with 10% FBS, at 37°C with 5% CO_2. All cell lines were tested for Mycoplasma and were found negative (Cellshipper Mycoplasma test; Bionique).

For cell proliferation experiments, cells were cultured in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc.) containing either 2 ng/mL TGF-β1 (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)] (4)-1*H*-pyrazole; Lilly designation HTS466284; Calbiochem), 10 μg/mL of 1D11, a pan-isoform, neutralizing TGF-β monoclonal antibody, or 13C4, a murine monoclonal isotype control antibody (kindly provided by Genzyme Inc.). Cells were trypsinized and counted using a Coulter counter at 24 and 48 hours post-treatment.

Mink lung cell luciferase assay

To measure secreted active and latent TGF-β in conditioned media, luciferase induction by conditioned media was measured in the mink lung epithelial cells transfected with truncated plasminogen activator inhibitor-1 promoter fused to the firefly luciferase reporter gene as previously described (29). Active TGF-β was measured directly in untreated samples, whereas total TGF-β (active and latent) was measured following heat activation at 80°C for 5 minutes (30). Unconditioned media was used as a control in all experiments, which was subtracted, and each condition was repeated in the presence of pan-specific TGF-β neutralizing antibody to confirm specificity. All experiments were carried out in triplicate, and results represent the mean measured value per 10^6 cultured cells.

Neurosphere assay

Cells were diluted in serum-free growth medium (1,000 cells/mL) and plated in 500 μL in non–adherent 24-well plates (Corning-Costar). Cells were fed with 125 μL of serum-free growth medium every other day for 14 days. The culture medium consisted of serum-free DMEM/F12 (Invitrogen) supplemented with 10 U/mL heparin (Sigma-Aldrich), 2% B27 (Invitrogen), human recombinant fibroblast growth factor 2 (FGF-2, 20 ng/mL; Sigma-Aldrich), and epidermal growth factor (EGF, 20 ng/mL; Sigma-Aldrich). After 14 days, spheres were measured, and those >100 μm were counted as a neurosphere-forming unit. To generate secondary neurospheres, primary neurospheres were gently centrifuged, mechanically dissociated, and then incubated with trypsin at 37°C for 5 minutes. After centrifugation and washing with PBS, cells were diluted using neurosphere media to 1,000 cells/mL and plated onto non–adherent 24-well plates, as described above. Spheres were counted and measured from 6 different wells for each experiment. Data on the neurosphere number and size are the average ± SE of 3 independent experiments consisting of 3 replicates.

Clonogenic assay

To assess clonogenic survival of cells in monolayer culture, human and murine glioma cell lines were grown for 48 hours to 70% confluence, wherein cells were incubated with serum replacement media containing 400 nmol/L of LY364947 kinase inhibitor or 10 μg/mL pan-specific TGF-β-neutralizing antibody 1D11 or control antibody 13C4 for 48 h before and 3 h post radiation exposure. Cells were irradiated with 1 to 8 Gy using a Varian Clinac 2300 C/D linear accelerator (Varian), trypsinized 3 hours post-irradiation, and plated in triplicates at 3 dilutions into 6-well cell culture plates in serum-containing media. Colonies were allowed to grow for 10 to 12 days followed by fixing and staining with crystal violet. Colonies containing >50 cells were counted to determine percent survival and the number of colonies obtained from 3 replicates was averaged for each treatment. These mean values were corrected according to plating efficiency of respective controls to calculate cell survival for each dose level.

To assess clonogenic survival of cells in neurosphere culture, neurospheres were cultured for 14 days under the neurosphere conditions described above, then treated with 400 nmol/L of LY364947 kinase inhibitor for 48 before and 3 hours post irradiation (2 Gy). Cells were centrifuged, media aspirated, and plated in triplicate at 3 dilutions into 6-well cell culture plates in serum-containing media. The remainder of the assay was conducted as described above.

Western blotting

To examine the DNA damage response and TGF-β signaling, 5×10^5 cells were grown in complete media for 48 hours, followed by LY364947 treatment (400 nmol/L) in 10% SRM for 24 hours. The cells were irradiated with 5 Gy and lysed after 1 hour, or treated with 500 pg/mL TGF-β and lysed after 30 minutes. Thereafter, the extracts were subjected to immunoblot analysis with one of the primary antibodies: phospho-Smad2 on serine 465/467 at 1:500 (clone 138D4, CAT#3108, Cell Signaling), Smad2/3 at 1:500 (CAT#610842, Becton Dickinson Transduction Laboratories), phospho-p53 on serine 15 at 1:500 (CAT#92845, Cell Signaling), p53 at 1:500 (Clone Ab-8, CAT#MS-738-P, Neomarkers and CAT#554157, Becton Dickinson Biosciences), ATM serine1981 phosporylation at 1:500 (CAT#2152-1, Epitomics), and ATM, clone 2C1 at 1:500 (CAT#GTX70103, GeneTex). Protein estimation was carried out using the BCA protein assay kit (Pierce). One hundred microgram of protein was electrophoresed on a 4% to 15% gradient gel (BioRad) and transblotted on polyvinylidene difluoride (PVDF) Immobilion-FL membrane (Millipore Corporation). The membrane was blocked in blocking buffer and probed with a primary antibody. The membrane was washed 3 times for 10 minutes with 0.1% Tris-buffered saline Tween 20 (TBST), followed by incubation with secondary antibodies (goat anti-mouse, CAT#926-32220 and goat anti-rabbit, CAT#926-32211, Odyssey) for 1 hours at room temperature. The membrane was washed 3 times for 10 minutes with TBST 0.1% and scanned on the Odyssey LICOR system. Using ImageJ 1.45s software (NIH), the raw integrated density was measured for each band of the protein of interest in all 3 cell lines. After correction for loading using actin, the ratio of phosphorylated to total protein was determined, normalized to the control group, and represented as fold change from the control group. Representative figures are displayed in grayscale.

Comet assay

The persistence of DNA damage following fractionated irradiation on neurosphere cultures was assessed by Comet-Assay. Neurospheres were cultured and treated with 400 nmol/L of LY364947 kinase inhibitor as described above and irradiated with 2 Gy for 3 consecutive days beginning on day 10 in culture. Neurospheres were dissociated and harvested 24 h following the third fraction. Single-cell gel electrophoresis at 19 V (300 mAMP, 40 min) was conducted by Alkaline CometAssay (Trevigen) according to the manufacturer's instructions. SYBR Green-stained DNA comets were imaged at ×100 magnification and the extent of DNA breaks was quantified as tail moment using CometScore software.

Immunofluorescence

GL261, U251, and U87MG cells (2×10^4) were grown in chamber slides in complete media for 48 hours, followed by LY364947 treatment (400 nmol/L) in 10% SRM for 24 hours before 2 Gy radiation. GL261-derived neurospheres, tumor cryosections or cells prepared as described above 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 hours, incubated with mouse detective antigen (Biocare Medical) for an additional 3 h for murine glioma tumors, and incubated with a mouse monoclonal γH2AX antibody (clone JBW301, Upstate Biotechnology; Charlottesville, VA) at 1:500, rabbit monoclonal phospho-serine 465/467 Smad2 antibody at 1:100 (clone 138D4, CAT#3108, Cell Signaling), goat polyclonal CXCR4antibody (CAT# ab1670, Abcam) at 1:300, or rabbit monoclonal Notch1-antibody (CAT# ab8925, Abcam) at 1:100, overnight at 4°C, followed by washes and incubation with Alexa-488- or Alexa-594-labeled anti-mouse/anti-rabbit/anti-goat secondary antibodies (Molecular Probes) for 1 hours at room temperature. Specimens were counter-stained 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× objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epifluorescence. All images were acquired with a CCD Hamamatsu Photonics (Herrsching am Ammersee, Germany) monochrome camera at 1392 × 1040 pixel size, 12 bits per pixel (bpp) depth and assembled as false-color images using the Metamorph imaging platform (Molecular Devices, Inc.). Foci were enumerated as previously described (31).

In vivo tumor studies

Animal studies were conducted using protocols that had undergone institutional review and approval. Female C57/BL6 mice, age 6 to 8 weeks, obtained from Taconic were used for animal experiments. Animals were housed in a temperature-controlled animal care facility with a 12-hour light–dark cycle

and allowed chow and water *ad libitum*. GL261 cells (10^6) were injected into the right flank of mice and allowed to grow. After the tumors reached an average size of 140 mm^3, animals ($n = 10$ in each group) were randomized to receive 1D11 TGF-β neutralizing antibody or 13C4 control antibody (10 mg/kg, intraperitoneal injection). Twenty-four hours later, tumors were irradiated with a dose of 6 Gy using a Varian Clinac 2300 C/D linear accelerator 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 tumor volumes were measured with a caliper. Tumor volumes were calculated as length × width^2 × 0.52 with all measurements in millimeter. Animals were sacrificed when tumors reached 10 × 10 mm in two dimensions. Two hours before sacrifice, animals were injected with pimonidazole (60 mg/kg intraperitoneal, HPI). Tumors were harvested and portions were formalin fixed and frozen in O.C.T. (Sakura Tissue-Tek). All animal experiments were carried out in accordance with guidelines specified by New York University's institutional animal care and use committee. For analysis, each tumor was normalized to its pretreatment volume.

Statistical analysis

The significance of the difference between mean values was calculated by conducting a 2-way Student *t* test. The significance of the difference between the mean values for graded doses of radiation in clonogenic assays, neurosphere formation, and γH2AX foci quantification was calculated by conducting a one-way ANOVA test with Tukey *post hoc* test. ANOVA with the Student Newman–Keuls Multiple Comparison posttest was used to determine significance between *in vivo* tumor growth delay measured by time-to-reach 3 times the pretreatment volume. A P value of <0.05 was considered significant.

Results

Inhibition of TGF-β radiosensitizes glioma cells

In serum-free conditions, glioma murine GL261 and human U251 and U87MG cell lines produced comparable amounts of active and latent TGF-β; GL261 162.3 ± 11.7 pg/mL and 477.6 ± 67.8 pg/mL; U251 123.1 ± 15.0 pg/mL and 420.5 ± 97.9 pg/mL; U87MG 82.5 ± 18.6 pg/mL and 350.5 ± 144.2 pg/mL, respectively, and showed intact TGF-β signaling through Smad phosphorylation (Fig. 1A). Murine GL261 and human U251MG cells were not responsive to growth modulation by exogenous TGF-β or blockade of endogenous TGF-β signaling by the addition of the TGF-β small-molecule inhibitor LY364947. U87MG cells were growth inhibited by addition of TGF-β by 19.7% ± 8.8% at 24 h and 55.3% ± 9.3% at 48 h, an effect that was reversed by the addition of the TGF-β small-molecule inhibitor LY364947 (data not shown).

Regardless of TGF-β-mediated growth modulation, addition of the TGF-β inhibitor LY364947 significantly increased the radiosensitivity of all 3 cell lines as measured in clonogenic

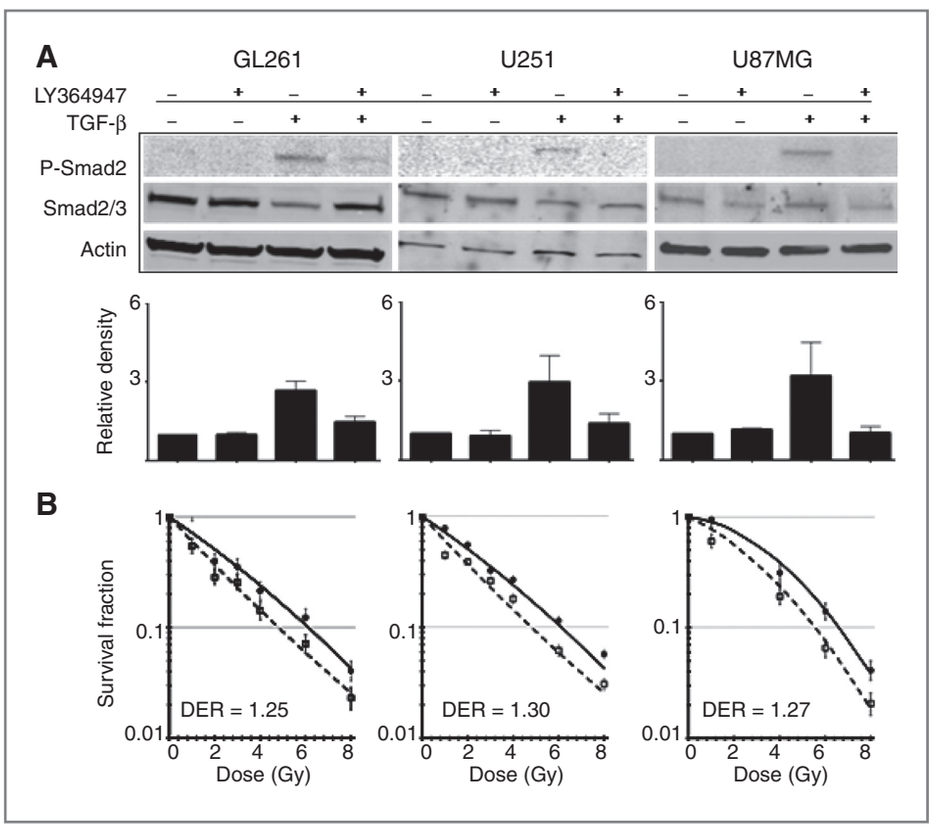

Figure 1. TGF-β inhibition radiosensitizes glioma cells independent of effects on proliferation. A, human (U251 and U87MG) and mouse (GL261) glioma cell lines respond to exogenous TGF-β1 (2 ng/mL), as measured by Smad phosphorylation, an effect that was blocked with the addition of the TGF-β type 1 receptor kinase inhibitor, LY364947. Representative immunoblots of 3 replicates and bar plots of densitometry quantitation of mean + SE are shown. B, clonogenic assay of GL261, U251, and U87MG glioma cells with (open symbols) and without (closed symbols) pretreatment with LY364947 24 hours before radiation exposure shows that TGF-β inhibition significantly radiosensitizes all 3 glioma cell lines. The DER at 10% survival is between 1.25 and 1.30. Mean ± SD values of triplicate determinations are shown. GL261, $P = 0.04$; U251, $p = 0.03$; U87MG, $P = 0.03$, ANOVA with Tukey *post hoc* test.

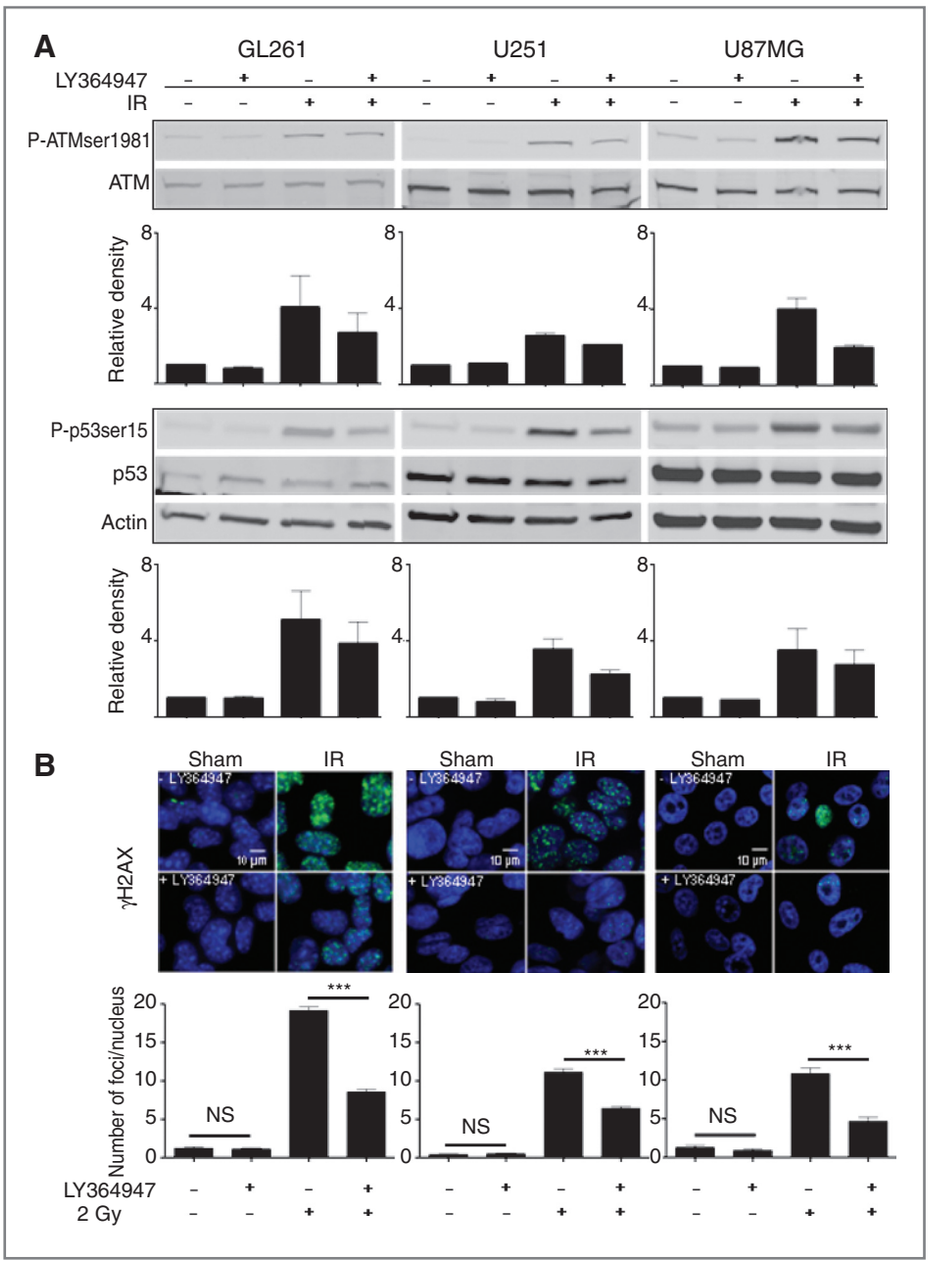

Figure 2. Radiosensitization of glioma cells by TGF-β inhibition correlates with reduction of the DNA damage response. A, aspects of the DNA damage response of the murine GL261 and human U251 and U87 MG glioma cell lines assessed by immunoblotting cell lysates obtained 30 minutes after 2 Gy of ionizing radiation. Pretreatment with TGF-β inhibitor LY364947 decreased IR-induced ATM phosphorylation at serine 1981 and p53 phosphorylation at serine 15 in all 3 cell lines. Representative immunoblots of 2 to 3 replicates and bar plots of densitometry quantitation of mean ± SE are shown. B, TGF-β inhibition with LY364947 significantly decreased γH2AX foci (green) in murine GL261 and human U251 and U87 MG glioma cells. Nuclei are counterstained with DAPI (blue). Quantification of γH2AX foci shown below each panel reveal a significant reduction in the number of radiation-induced γH2AX foci with LY354947 in GL261 cells (19.2 ± 0.5 vs. 8.6 ± 0.4) U251 cells (11.1 ± 0.4 vs. 6.4 ± 0.2) and U87MG cells (10.8 ± 0.7 vs. 4.7 ± 0.6). ***, $P < 0.0001$; ANOVA.

assay (Fig. 1B; GL261 $P = 0.04$, U251 $P = 0.03$, U87MG $P = 0.03$, ANOVA with Tukey *post hoc* test). The dose-enhancement ratio (DER) at 10% cell survival was 1.25 to 1.30, which indicates that 25% less dose was necessary to kill 90% of the cells by radiation when TGF-β signaling was blocked. In addition, LY364947 significantly decreased radiation-induced phosphorylation of ATM Ser1981 and P53 Ser15 (Fig. 2A). Consistent with prior studies in human and mouse epithelial cells (25, 28), radiosensitization was correlated with significantly fewer γH2AX foci, a marker of DNA damage response (Fig. 2B). These data indicate that TGF-β inhibition in glioma cells compromises the response to DNA damage and increases radiosensitivity.

There are 3 pharmacologic routes to blocking TGF-β: neutralizing the ligand, inhibiting expression, and truncating the signaling cascade (32). The pharmacokinetic properties of antibody and small-molecule kinase inhibitors result in considerable differences in the duration of TGF-β signal modulation. Several TGF-β neutralizing antibodies that are in clinical development have shown safety and efficacy in fibrotic disorders (32, 33). We compared *in vitro* efficacy of TGF-β ligand captured using 1D11 pan-TGF-β neutralizing antibodies to that of LY364947 in monolayer GL261 cells. Pretreatment with pan-specific TGF-β neutralizing antibody, 1D11, produced the same level of radiosensitization as LY364947 (Fig. 3A; $P = 0.04$, ANOVA with Tukey *post hoc* test).

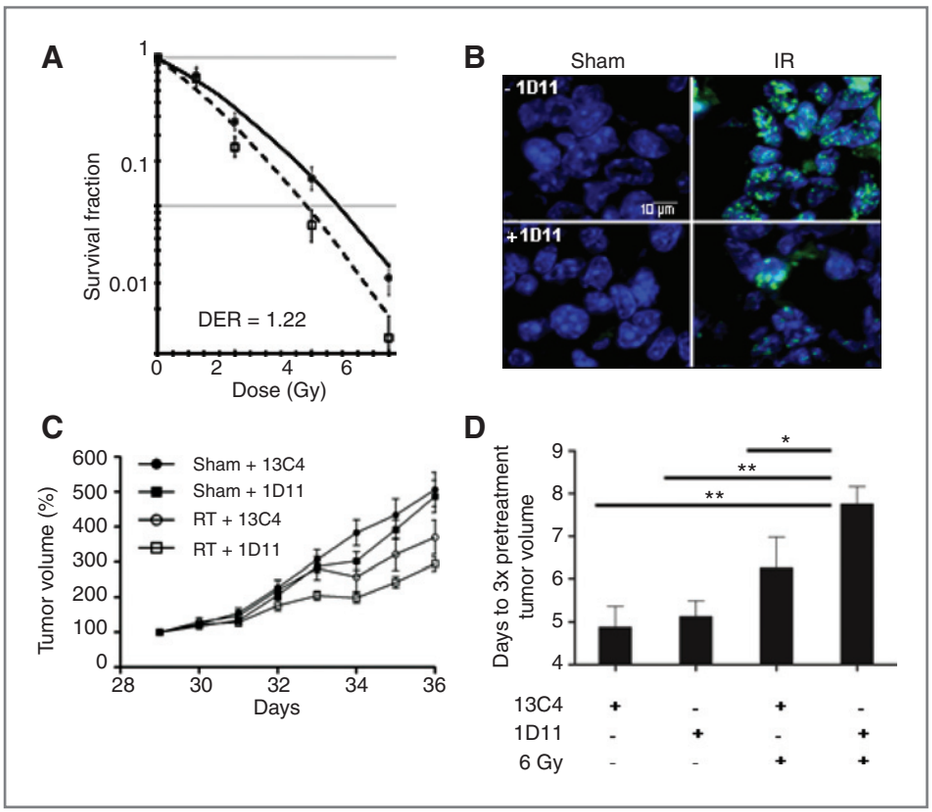

Figure 3. Treatment with 1D11 pan-specific TGF-β neutralizing antibody radiosensitizes glioma cells *in vitro* and *in vivo*. A, inhibition of TGF-β using a pan-specific monoclonal antibody, 1D11, resulted in similar level of radiosensitization (1.22 at 10% cell survival) as seen with the use of the small-molecule inhibitor LY364947. $P = 0.04$, ANOVA with Tukey *post hoc* test. B, treatment with 1D11 neutralizing antibody resulted in decreased γH2AX immunofluorescence (green) 1 hour after 2 Gy compared with irradiated mice receiving control antibody 13C4. Nuclei are counterstained with DAPI (blue). C, a single intraperitoneal injection of 1D11 antibody 24 hours before irradiation (6 Gy) of GL261 flank tumors resulted in greater tumor growth delay compared with mice receiving 13C4 control antibody. The *y*-axis represents tumor volume normalized to pretreatment volume. RT/13C4 versus RT/1D11, $P < 0.05$; RT/1D11 versus Sham/1D11, $P < 0.01$; ANOVA with Newman–Keuls multiple comparison posttest. D, the time-to-reach 3 times pretreatment volume was significantly increased by 1D11 treatment compared with antibody control 13C4. *, $P < 0.05$; **, $P < 0.01$, ANOVA with Newman–Keuls multiple comparison posttest.

To test whether increased radiosensitivity conferred therapeutic benefit, we established flank tumors of GL261 cells in C57bl mice. A single intraperitoneal injection of 1D11 antibody (10 mg/kg) did not affect tumor growth rate of established tumors (~150 mm^3) compared with control–antibody-treated mice. Immunohistochemical detection of γH2AX in tumors harvested 1 h after radiation was reduced in mice treated with 1D11 (Fig. 3B). Consistent with inhibition of DNA damage recognition, the tumor growth delay of mice treated with 1D11 injected 24 h before a single 6 Gy fraction was significantly increased compared with IR alone (Fig. 3C and D).

Radiosensitivity of GL261 neurosphere formation

Tumor regrowth is hypothesized to be, in large part, due to the relative response of GIC (5); therefore, we next examined the effect of TGF-β on GL261 neurosphere-forming capacity as a surrogate of GIC. Addition of LY364947 to GL261 neurosphere cultures did not affect either primary or secondary neurosphere-forming capacity. Irradiation (2 Gy) of monolayer GL261 cells significantly decreased primary neurosphere-forming capacity by 28% ($P < 0.001$; ANOVA). Addition of TGF-β inhibitor LY364947 decreased primary neurosphere formation by another 47% for a total reduction of 75% (Fig. 4A; $P < 0.001$; ANOVA). Surprisingly, irradiation of primary neurospheres did not affect secondary neurosphere formation. However, LY364947-treated, irradiated primary neurosphere cultures showed significantly decreased secondary neurosphere-forming capacity, leading to a 68% reduction (Fig. 4B; $P < 0.001$; ANOVA).

Given that secondary neurosphere formation, a measure of GIC self-renewal, was resistant to radiation but the majority of cells in a neurosphere are not GIC, we explored whether both populations were afforded the same degree of resistance by measuring clonogenic survival of GL261 cells dissociated from treated neurospheres. Consistent with monolayer cultures, radiation decreased colony-forming efficiency, and addition of TGF-β inhibitor LY364947 further increased the radiosensitivity of these cells by a similar magnitude to the effect seen in GL261 bulk culture. Irradiation (2 Gy) reduced the surviving fraction by 43% and the addition of LY354947 further reduced clonogenic survival by an additional 20% (Fig. 4C). Thus, non-

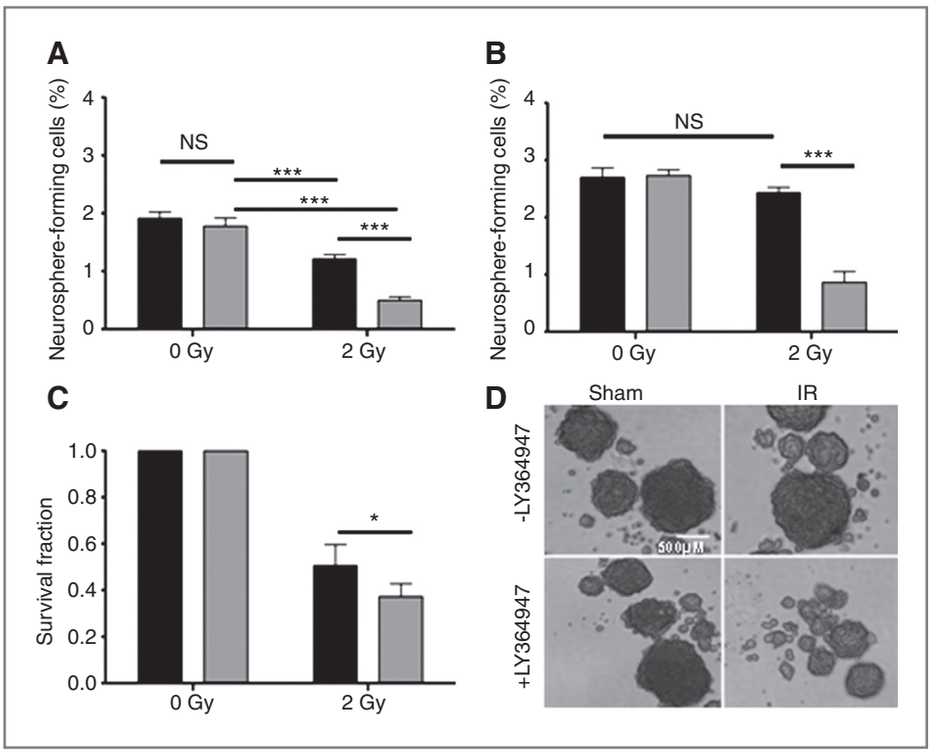

Figure 4. TGF-β inhibition in conjunction with radiation decreases GL261 neurosphere-forming capacity and radiosensitizes neurosphere-derived cells. GL261 murine glioma cells were cultured under neurosphere conditions. A, treatment with LY364947 (24 hours, 400 nmol/L) alone had no effect on primary neurosphere formation. Irradiation (2 Gy) decreased primary neurosphere-forming capacity by 28%, and LY364947 treatment for 24 hours before irradiation decreased neurosphere formation by an additional 47%, resulting in 75% fewer neurospheres. B, treatment with LY364947 (24 hours, 400 nmol/L) alone had no effect on secondary neurosphere formation. Irradiation of primary neurospheres had no effect on secondary neurosphere-forming capacity, yet LY364947 treatment of primary neurospheres before irradiation decreased secondary neurosphere formation by 68%. C, pretreatment with LY364947 for 24 hours decreased neurosphere-derived clonogenic cell survival after irradiation (2 Gy). After 2 Gy irradiation, the survival fraction of untreated neurospheres was reduced by 43%, whereas the survival fraction of neurospheres treated with 2 Gy and LY354947 was further reduced by an additional 20%. D, representative images of neurospheres from sham, control-treated, sham, LY364947-treated, 2 Gy, control-treated, and 2 Gy, LY364947-treated group. Data are means ± SD of triplicate determinations and representative of 3 experiments. NS, not significant; *, $P < 0.05$; ***, $P < 0.0001$; ANOVA.

GICs were not afforded protection from IR in neurosphere culture. These data support the contention that GIC are specifically protected from radiation (8); however, this is conditional, that is, specific to neurosphere culture.

TGF-β inhibition reduces DNA damage response in GL261 neurospheres and radiosensitizes the GIC population. We hypothesized that lack of radiation effect on secondary neurosphere formation, which is indicative of self-renewal, would be reflected in the DNA damage response. To test this hypothesis, γH2AX foci induced by 2 Gy were measured at 30 minutes post-radiation in GL261 neurospheres (Fig. 5A). Surprisingly, we observed that the number of foci per cell was 6 in neurosphere-derived cells versus 19 in monolayer culture at the same time after the same dose. The difference between monolayer and neurosphere culture could be a failure to recognize damage, a more effective DNA damage response, or both (34). Nonetheless, addition of TGF-β inhibitor LY364947 in neurosphere culture reduced radiation-induced γH2AX foci by 5.2-fold (6.2 foci/nucleus compared with 1.2 foci/nucleus; Fig. 5B), indicating that TGF-β inhibition compromised the molecular recognition of DNA damage in this population, as well as in GL261 bulk culture.

These data indicate that DNA damage recognition is compromised and that fractionation, which is standard in radiotherapy, would amplify this effect and further compromise DNA repair. To test this hypothesis, we exposed GL261 neurospheres to 3 daily fractions of 2 Gy with or without LY364947 and conducted comet assays to evaluate unresolved DNA damage at 24 h after the final dose (Fig. 5C). Radiation significantly increased the mean tail moment of cells isolated from neurospheres. The mean of those treated with radiation and LY364947 was 19.8 ± 17 SD compared with 6 ± 6 SD for fractionated radiation alone. Less than 5% of non-irradiated cells had tail moments more than 10, compared with 17% for those treated with fractionated radiation and 64% of those from cells treated with the small-molecule TGF-β signaling inhibition and fractionated radiation. These data indicate that TGF-β inhibition not only compromises DNA damage recognition but also prevents DNA repair.

Previously, our studies showed that *Tgfb1* null cells fail to mount the full DNA damage response and were radiosensitive (25), as was observed by inhibiting TGF-β signaling in monolayer GBM cultures. Because GIC appeared to be radioresistant, but were sensitized by TGF-β blockade, we hypothesized that increased TGF-β production and/or activity could

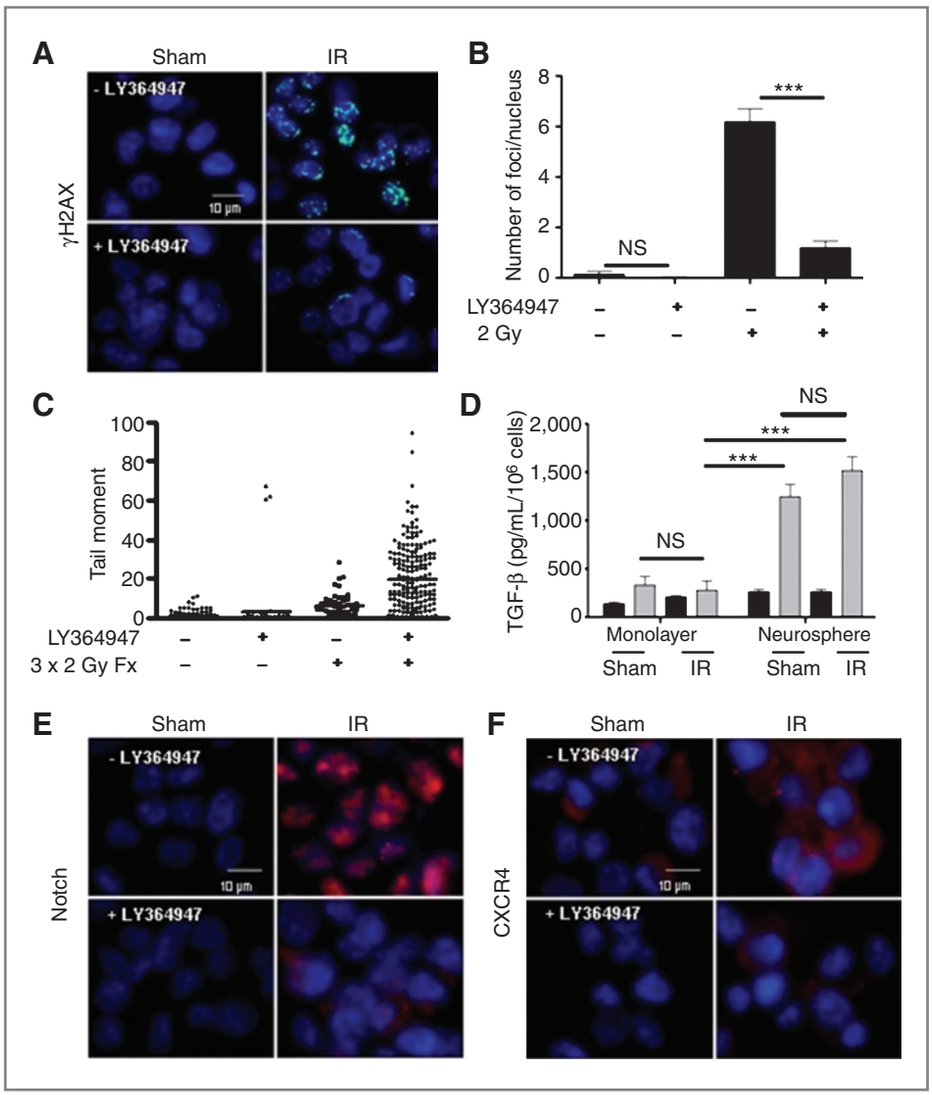

Figure 5. TGF-β inhibition affects DNA damage response and self-renewal pathways upregulated by ionizing radiation. A, LY364947 pretreatment for 24 hours before 2 Gy decreased radiation-induced γH2AX foci in GL261 cells derived from neurosphere. B, quantification revealed an 81% decrease in the number of radiation-induced γH2AX foci with LY364947 treatment (6.2 ± 0.5 vs, 1.2 ± 0.3). C, comet assay was used to determine that LY364947 inhibited DNA repair following fractionated (3 × 2 Gy) daily radiation exposure of neurosphere cultures. Data shown are representative of 3 experiments. Comets measured for control, $N = 317$; LY364947 treated, $N = 74$; fractionated 3 × 2 Gy, $N = 52$; LY364947 treated and fractionated 3 × 2, $N = 197$. D, TGF-β production by GL261 cells was measured in conditioned media obtained following 48 hours in monolayer or neurosphere growth conditions. GL261 cells grown under neurosphere conditions produced 3.7-fold more total and 1.9-fold more active TGF-β per cell than cells grown in monolayer culture. ***, $P < 0.0001$, single-tailed unpaired *t* test. E, inhibition of TGF-β with LY364947 also blocked radiation-induced Notch1 immunofluorescence (red), and CXCR4 immunofluorescence (red; F), in GL261 cells dissociated from neurospheres 3 hours after irradiation with 2 Gy. Nuclei are counterstained with DAPI (blue).

underlie their phenotype. Active and total TGF-β levels in media conditioned for 72 hours from GL261 monolayers or neurospheres were measured using the mink lung epithelial cell luciferase assay. As previously reported, the TGF-β2 isoform accounts for 85% of total TGF-β produced by cells grown under either condition, determined by isoform-specific neutralizing antibodies (data not shown). GL261 cells in neurosphere culture produced significantly more (3.7- ± 1.4-fold, $P < 0.001$, ANOVA) total TGF-β and a trend toward more active TGF-β (1.9- ± 1.5-fold, $P > 0.05$) than GL261 cells in monolayer culture (Fig. 5D). Irradiation before treatment with conditioning media under either of the culture conditions did not significantly affect TGF-β levels. Thus, increased production of TGF-β by GL261 neurospheres could be protective as evidenced by a more effective molecular response to radiation-induced DNA damage leading to radiation resistance.

To test the idea that TGF-β inhibition affects GIC self-renewal following IR, we examined CXCR4 and Notch1, which have been implicated in GIC self-renewal pathways (35, 36). Irradiation (2 Gy) of primary neurospheres significantly induced both markers, measured 3 hours following radiation treatment, which was blocked by TGF-β inhibition with LY364947 (Fig. 5E and F). Thus, we concluded that GIC are protected from radiation-induced cell kill by increased TGF-β production under conditions approximating the niche (i.e., neurosphere culture), which promotes effective DNA damage response and self-renewal via CXCR4 and Notch 1. Inhibition of TGF-β signaling compromises both mechanisms.

Discussion

GBM is a cancer characterized by a high degree of radioresistance, evidenced by inevitable local and/or disseminated recurrence. Our study indicates that high TGF-β levels confer resistance for both GIC and more differentiated tumor cells to DNA damage. Tumors have been described as "wounds that do not heal", whose considerable similarities with the process of wound healing include TGF-β activity. Ionizing radiation is

certainly another source of injury to tumor cells and tumor microenvironment, as well as surrounding normal tissue (20–22, 37). Pharmaceutical TGF-β inhibition circumvents this microenvironment-mediated protection by compromising DNA damage recognition and, therefore, repair, as evidenced by increased clonogenic cell death and unrepaired DNA measured by comet assay. The potential for benefit is likely even higher *in situ*, as there are multiple sources of TGF-β and multiple modes of action of TGF-β besides the DNA damage response on both cell behavior and tumor microenvironment. Indeed, several studies have shown potential therapeutic benefit for TGF-β inhibition in preclinical glioma models, including antiangiogenesis (38) and anti-invasion (39, 40).

Our previous work showed that genetic depletion of TGF-β1 compromises the DNA damage response *in vivo* and *in vitro* (25, 26), and we have now shown *in vitro* and *in vivo* radiosensitization with TGF-β inhibitors in breast cancer models (28). The negative impact of TGF-β on response to radiation therapy has been observed in multiple tumor types, further illustrating the well-established pleotropic effects of this cytokine in the tumor microenvironment. The addition of TGF-β inhibitors improves radiation response in preclinical models of GBM (41, 42). Zhang and colleagues specifically reported that the addition of the small-molecule inhibitor of TGF-β receptor type I and II kinase, LY2109761, to the current standard of care treatment, radiation and the oral alkylating agent temozolomide, provided benefit. In addition to radiosensitization and tumor growth delay, TGF-β-signaling blockade had antiangiogenic and antimigration effects as well. Mengxian and colleagues similarly reported radiosensitization, tumor growth delay, and improved survival with the addition of the same small-molecule inhibitor of TGF-β, LY2109761, without combining with temozolomide. They further showed that either TGF-β inhibition or radiation decreased self-renewal of glioma stem-like cells in a neurosphere assay, and a greater decrease was noted when these were combined. Our study provides an explanation of these findings in that autocrine TGF-β potentiates an effective molecular DNA damage response as well as self-renewal.

TGF-β inhibitors are already in phase II/III clinical trials for fibrosis (32, 33). Phase I/II clinical studies using the antisense oligonucleotide AP-12009 (Antisense Pharma, Regensberg, Germany) to target TGF-β2 in recurrent or refractory WHO grade III or IV glioma showed prolonged survival when compared with historical controls (43). More recently, a randomized phase IIb study of AP-12009 in patients with recurrent or refractory GBM or anaplastic astrocytoma showed tumor control rate superiority of lower dose AP-12009 over standard chemotherapy in anaplastic astrocytoma and comparable survival in GBM, with lower rates of toxicity observed with AP-12009 compared with chemotherapy (44).

Our study includes several aspects that add significantly to the growing body of evidence indicating that TGF-β is a therapeutic target in GBM. First, we used both a small-molecule inhibitor of TGF-β type I receptor kinase, LY364947, as well as a pan-specific TGF-β neutralizing antibody, 1D11, to target the TGF-β pathway in combination with IR. Potential advantages seen with clinical use of neutralizing antibodies to TGF-β or its receptor include their longer half-life and potentially more consistent inhibition than small-molecule inhibitors, as well as targeting of all 3 TGF-β isoforms that could be beneficial by affecting not only tumor cells but also the tumor microenvironment. Although the blood–brain barrier does prevent antibody entry into normal brain tissue, the situation is much more complex in the setting of GBM, in which the tumor itself can modulate the blood–brain barrier's permeability (45). Indeed, the pan-specific TGF-β neutralizing antibody 1D11 has been shown in preclinical orthotopic models to concentrate intratumorally (46). Furthermore, the recent success with bevacizumab (Avastin, an anti-VEGF monoclonal antibody), in both recurrent and newly diagnosed GBM, highlights the feasibility of therapeutic antibodies in CNS tumors (reviewed in ref. 47). Although outcomes have improved, concern over the altered pattern of relapse in patients with bevacizumab-treated GBM, characterized by distant infiltration of the brain by tumors that show increased invasiveness, has emerged (48). Combination therapy with strategies to inhibit invasion have been proposed, and the TGF-β pathway is a logical approach, given the documented role TGF-β plays in glioma migration and invasion (11).

The magnitude of radiosensitization seen in the current study (DER ~1.25 by clonogenic assay) must be taken into context of a disease as difficult to treat as GBM. The DERs currently reported are well within the level of radiosensitization seen in models of glioma. Zheng and colleagues used siRNA silencing of TNF receptor-associated Factor 2 (TRAF2) to radiosensitize U251 glioma cells *in vitro* with a DER of 1.2 to 1.39 (49). Golding and colleagues reported radiosensization with DERs of 1.6 to 2.1 *in vitro* using ATM kinase inhibitors KU-55933 in U87MG glioma cells (50) and KU-60019 in U1242 glioma cells (51). Our study showed that ATM kinase activity is reduced with TGF-β inhibition. More importantly, Kil and colleagues showed a DER of 1.32 using U251 glioma cells treated with temozolomide (52). Considering that the addition of temozolomide to radiation therapy in the treatment of GBM was one of the largest breakthroughs in this disease in decades and is now considered the standard of care, radiosensitization of this magnitude reported here must be considered significant, particularly because the radiation sensitivity of GIC increased nearly 3-fold.

Debate exists about whether GIC are more (8) or less (53) radioresistant than the tumor cell population as a whole, but it is hypothesized that the GIC population contributes to the inevitable recurrence of GBM (54). Several recent studies have shown that the TGF-β pathway is important in GIC biology (17–19). Although we did not observe inhibition of neurosphere self-renewal with TGF-β inhibition alone (seen in several of the abovementioned studies), we found that TGF-β inhibition in combination with IR prevents self-renewal mediated by CXCR4 and Notch1. These data resolve the paradoxical literature as to GIC radiation sensitivity. We postulate that TGF-β production in the GIC niche is evidence of microenvironment-mediated resistance, and as such represents a very promising target to improve GBM radiotherapy and provide multifaceted benefits that could prevent GBM recurrence.

Disclosure of Potential Conflicts of Interest

S.M. Lonning is employed by Genzyme, Inc. as Senior Director, Oncology Research. No potential conflicts of interest were disclosed by the other authors.

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Provided reagents used in experiments: S.M. Lonning

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