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Resistance of glioblastoma initiating cells to radiation mediated by the tumor microenvironment can be abolished by inhibiting transforming growth factor- β (TGF 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 suggest that GIC are sensitive but the response is mediated by undefined factors in the microenvironment. GBM produce abundant transforming growth factor- β (TGF β), a pleiotropic 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 approximately 25% when treated with LY364947, a small molecule inhibitor of TGF β type I receptor kinase, prior to 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 primary or secondary neurosphere-forming capacity. IR decreased primary neurosphere formation by 28%, but did not reduce secondary neurosphere formation. In contrast, LY364947 prior to 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 to traditional culture, suggesting that TGF β production by GIC promotes the DNA damage response and self-renewal and 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 GBM patients.

Keywords

TGF β ; DNA damage response; radiosensitivity; glioblastoma

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Introduction

Glioblastoma multiforme (GBM) remains a significant therapeutic challenge and 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). While the exact origin of GBM (and other malignant brain tumors) is unknown, it is thought that a fraction of tumor cells have cancer stem cell characteristics (glioma initiating cells, GIC) and true tumorigenic potential (2, 3). Originally proposed twenty-five years ago (4), it is generally thought that the resistance of GIC contributes to the poor response to radiation and chemotherapy and inevitable tumor recurrence (reviewed in (5)). The exact mechanism of treatment resistance is unknown, but GIC intrinsic hyperactivation of the PI3K/Akt and PTEN pathways (6, 7) and increased activation of DNA damage checkpoint pathways (8) are thought to contribute.

Microenvironment can also contribute to radiation responses (reviewed in (9)). Jamal *et al.* 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 pleiotropic 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 (11)). Both TGF β 1 and TGF β 2 have been implicated in autocrine tumor growth regulation (12). TGF β 2 is overexpressed in gliomas (13). Higher levels of TGF β 1 have been found in anaplastic gliomas (WHO grade III) than in GBM (WHO grade IV), suggesting a potential role of TGF β 1 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 (15)) and neural stem cells specifically (16). TGF β has been implicated in GIC biology as well. Penuelas *et al.* showed that exposure of 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 *et al.* reported that autocrine TGF β contributes to the tumorigenicity of the GIC population by activation of Sox4 and Sox2 (18). More recently, Anido *et al.* 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 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 likely 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 β 1 (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 telangiectasia mutated (ATM), a protein 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 thought 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 present study is 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 to bulk populations produce more TGF β , whose inhibition significantly compromises both DNA damage response and self-renewal of GIC.

Materials/Methods

Cell Culture

The murine glioma, GL261 (obtained from National Cancer Institute-Frederick Cancer Research Tumor Repository; Frederick, MD; authenticated in 2012 by Idexx Radil, West Sacramento, CA) and human glioma U251 (generous gift of Dr. Kevin Camphausen; authenticated in 2010 by Idexx Radil) cells were cultured in Dulbecco's Modified Eagle Medium GlutaMAX™ (Gibco; Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; St. Louis, MO) and 1% pyruvate (Gibco; Carlsbad, CA). Human glioma U87MG (obtained from ATCC in 2011) cells were cultured in Eagle's Minimum Essential Medium (Gibco; Carlsbad, CA) with 10% fetal bovine serum, at 37°C with 5% CO₂. All cell lines were tested for Mycoplasma and were negative (Cellshipper Mycoplasma test; Bionique, Saranac Lake, NY).

For cell proliferation experiments, cells were cultured in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc., Carlsbad, CA) containing either 2 ng/ml TGF β 1 (R&D Systems; Minneapolis, MN), 400 nM small molecule inhibitor of the TGF β type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)](4)-1H-pyrazole; Lilly designation HTS466284; Calbiochem; St. Louis, MO), 10 μ g/ml of 1D11, a pan-isoform, neutralizing TGF β monoclonal antibody, or 13C4, a murine monoclonal isotype control antibody (kindly provided by Genzyme Inc.; Framingham, MA). Cells were trypsinized and counted using a Coulter counter at 24 h and 48 h 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 five 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 conditions were performed in triplicate, and results represent the mean measured value per 10⁶ cultured cells.

Neurosphere Assay

Cells were diluted in serum-free growth medium (1000 cells/ml) and plated in 500 μ l in non-tissue culture coated 24-well plates (Corning-Costar; Lowell, MA). Cells were fed with 125 μ l of serum-free growth medium every other day, for 14 days. The culture medium consisted of serum-free DMEM/F12 (Invitrogen, Grand Island, NY) supplemented with 10 U/ml heparin (Sigma-Aldrich; St. Louis, MO), 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 μ m 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 five minutes. After centrifugation and washing with PBS, cells were diluted using neurosphere media at 1000 cells/ml and plated into non-tissue culture coated 24-well plates, as above. Spheres were counted and measured from 6 different wells per experiment. The neurosphere number and size data shown is the average + S.E. of three independent experiments consisting of three replicates.

Clonogenic Assay

To assess clonogenic survival of cells in monolayer culture, human and murine glioma cell lines were grown for 48 h to 70% confluence upon which cells were incubated with serum replacement media containing 400 nM of LY364947 kinase inhibitor or 10 μ g/ml pan-specific TGF β neutralizing antibody 1D11 or control antibody 13C4 for 48 hours before- and 3 hour post-radiation exposure. Cells were irradiated with 1-8 Gy using a Varian Clinac 2300 C/D linear accelerator (Varian; Palo Alto, CA), trypsinized 3 h post-irradiation and plated in triplicates at three dilutions into six-well cell culture plates in serum containing media. Colonies were allowed to grow for 10-12 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 three 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 neurosphere conditions described above, then treated with 400 nM of LY364947 kinase inhibitor for 48 hours before- and 3 hours-post irradiation (2 Gy). Cells were centrifuged, media aspirated, and plated in triplicate at three dilutions into six-well cell culture plates in serum containing media. The remainder of the assay was performed as above.

Western Blotting

To examine the DNA damage response and TGF β signaling, 5×10^5 cells were grown in complete media for 48 h, followed by LY364947 treatment (400 nM) in 10 % SRM for 24 h. 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. Then 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; Beverly, MA), Smad2/3 at 1:500 (CAT#610842, BD Transduction Laboratories; Lexington, KY), phospho-p53 on serine 15 at 1:500 (CAT#92845, Cell Signaling), p53 at 1:500 (Clone Ab-8, CAT#MS-738-P, Neomarkers; Fremont, CA and CAT#554157, BD Biosciences), ATM serine1981 phosphorylation at 1:500 (CAT#2152-1, Epitomics; Burlingame, CA) and ATM, clone 2C1 at 1:500 (CAT#GTX70103, GeneTex; San Antonio, TX). Protein estimation was carried out using the BCA protein assay kit (Pierce; Rockford, IL). One hundred μ g of protein was electrophoresed on a 4-15 % gradient gel (BioRad; Hercules, CA) and transblotted on PVDF ImmobilonTM-FL membrane (Millipore Corporation; Ballerica, MA). The membrane was blocked in blocking buffer and probed with a primary antibody. The membrane was washed 3 times for 10 min with 0.1% TBST, followed by incubation with secondary antibodies (goat anti-mouse, CAT#926-32220 and goat anti-rabbit, CAT#926-32211, Odyssey; Lincoln, NE) for 1 h at room temperature. The membrane was washed 3 times for 10 min with TBST 0.1% and scanned on the Odyssey LICOR system. Using ImageJ 1.45s software (National Institute of Health, USA) the raw integrated density was measured for each band

of the protein of interest in all 3 cells 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 DNA damage following fractionated irradiation on neurosphere cultures was assessed by CometAssay®. Neurospheres were cultured and treated with 400 nM of LY364947 kinase inhibitor as described above and irradiated with 2 Gy for three consecutive days beginning on day 10 in culture. Neurospheres were dissociated and harvested 24hrs following the third fraction. Single cell gel electrophoresis at 19V (300mAMP, 40 min) was preformed by Alkaline CometAssay® (Trevigen) according to the manufacturer's protocol. 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 h, followed by LY364947 treatment (400 nM) in 10% SRM for 24 h prior to 2 Gy radiation. GL261-derived neurospheres, tumor cryosections or cells prepared as above were fixed using 2% paraformaldehyde for 20 min at room temperature followed by permeabilization with 100 % methanol for 20 min at -20°C . Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 h, incubated with mouse detective antigen (Biocare Medical; Concord, CA) for an additional 3 hrs 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; Cambridge, MA) at 1:300, or rabbit monoclonal Notch1antibody (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; Eugene, OR) for 1 h 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; Burlingame, CA). Specimens were imaged using a 40X objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss; Wetzlar, Germany) 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.; Sunnyvale, CA). 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 6-8 weeks obtained from Taconic (Hudson, NY) were used for animal experiments. Animals were housed in a temperature-controlled animal care facility with a 12-h 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. Once tumors reached average 140mm^3 , animals ($n=10$ in each group) were randomized to receive 1D11 TGF β neutralizing antibody or 13C4 control antibody (10mg/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; Feldkirchen, Germany). 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 \times width² \times 0.52 with all measurements in mm. Animals were sacrificed when tumors reached 10mm x 10mm in two dimensions. Two hours before sacrifice, animals were injected with pimonidazole (60 mg/kg intraperitoneal, HPI; Burlington, MA). Tumors were harvested and portions were formalin fixed and frozen in O.C.T. (Sakura Tissue-Tek; Torrance, CA). All animal experiments were performed in accordance with New York University's institutional animal care and use committee. For analysis, each tumor was normalized to its pre-treatment volume.

Statistical Analysis

The significance of the difference between mean values was calculated by performing a two-way Students t-test. The significance of the difference between the mean values for graded doses of radiation in clonogenic assays, neurosphere formation, and γ H2AX foci quantification was calculated by performing a one-way ANOVA test with Tukey post-test. ANOVA with the Student's Newman-Keuls Multiple Comparison post-test was used to determine significance between *in vivo* tumor growth delay measured by time-to-reach three times pre-treatment 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 demonstrated intact TGF β signaling through Smad phosphorylation (Figure 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 24hrs and 55.3+9.3% at 48hrs, 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 three cell lines as measured in clonogenic assay (Figure 1B; GL261 *p*=0.04, U251 *p*=0.03, U87MG *p*=0.03, ANOVA with Tukey post-test). The dose enhancement ratio (DER) at 10% cell survival was 1.25-1.30, which indicates that 25% less dose was necessary to kill 90% of the cells by radiation when TGF β signaling was blocked. LY364947 also significantly decreased radiation-induced phosphorylation of ATM Ser1981 and P53 Ser15 (Figure 2A). Consistent with prior studies in human and mouse epithelial cells (25, 28), radiosensitization was correlated with a significantly fewer γ H2AX foci, a marker of DNA damage response (Figure 2B). These data indicate that TGF β inhibition in glioma cells abrogates the response to DNA damage and increases radiosensitivity.

There are three pharmacological 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 demonstrated 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. Pre-treatment with pan-specific TGF β neutralizing antibody, 1D11, produced the same level of radiosensitization as LY364947 (Figure 3A; $p=0.04$, ANOVA with Tukey post-test).

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 ($\sim 150 \text{ mm}^3$) compared to control antibody treated mice. Immunohistochemical detection of γH2AX in tumors harvested 1 hr after radiation was reduced in mice treated with 1D11 (Figure 3B). Consistent with inhibition of DNA damage recognition, the tumor growth delay of mice treated with 1D11 injected 24 hr before a single 6 Gy fraction was significantly increased compared to IR alone (Figure 3C-D).

Radiosensitivity of GL261 neurosphere formation

Tumor regrowth is thought to be, in large part, due to the relative response of GIC (5), hence 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% (Figure 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 (Figure 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 asked 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 that effect seen in GL261 bulk culture. Irradiation (2 Gy) reduced the surviving fraction by 43% and the addition of LY364947 further reduced clonogenic survival by an additional 20% (Figure 4C). Thus, non-GICs were not afforded protection from IR in neurosphere culture. These data support the contention that GIC are specifically protected from radiation (8), but that this is conditional, i.e. specific to neurosphere culture.

TGF β inhibition reduces DNA damage response in GL261 neurospheres and radiosensitizes the GIC population—We speculated 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, γH2AX foci induced by 2 Gy were measured at 30 minutes post-radiation in GL261 neurospheres (Figure 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 either a failure to recognize damage, or 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 vs 1.2 foci/nucleus) (Figure 5B), indicating that TGF β inhibition compromised the molecular recognition of DNA damage in this population, as well as GL261 bulk culture.

These data suggest that DNA damage recognition is compromised and that fractionation, which is standard in radiotherapy, would amplify this effect and compromise DNA repair.

To test this, we exposed GL261 neurospheres to 3 daily fractions of 2 Gy with or without LY364947 and performed comet assays to evaluate unresolved DNA damage at 24 hr after the final dose (Figure 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 S.D. compared to 6 ± 6 S.D. for fractionated radiation alone. Less than 5% of non-irradiated cells had tail moments above 10, compared to 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 suggest that TGF β inhibition not only compromises DNA damage recognition but prevents DNA repair.

Our prior 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. Since GIC appeared to be radioresistant, but were sensitized by TGF β blockade, we speculated that increased TGF β production and/or activity could underlie their phenotype. Active and total TGF β levels in media conditioned for seventy-two hours from GL261 monolayers or neurospheres were measured using the mink lung epithelial cell luciferase assay. As expected, the TGF β 2 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 (Figure 5D). Irradiation before 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 three hours following radiation treatment, which was blocked by TGF β inhibition with LY364947 (Figure 5 E, 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 suggests 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 increase in therapeutic index 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 demonstrated potential therapeutic benefit for TGF β inhibition in preclinical glioma models, including anti-angiogenesis (38) and anti-invasion (39, 40).

Our previous work showed that genetic depletion of TGF β 1 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 recently been demonstrated in multiple tumor types, further illustrating the well-established pleiotropic effects of this cytokine in the tumor microenvironment. Addition of TGF β inhibitors improves radiation response in preclinical models of GBM (41, 42). Zhang *et al.* 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 anti-angiogenic and anti-migration effects as well. Mengxian *et al.* 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 demonstrated that either TGF β inhibition or radiation decreased self-renewal of glioma stem-like cells in a neurosphere assay, and a greater decrease when these were combined. Our study provides the 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, Regensburg, Germany) to target TGF β 2 in recurrent or refractory WHO grade III or IV glioma demonstrated prolonged survival when compared to 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 to chemotherapy (44).

Our study includes several aspects that add significantly to the growing body of evidence that TGF β is a therapeutic target in GBM. First, we use 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 three 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). Further, 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 (47)). Although outcomes have improved, concern over the altered pattern of relapse in bevacizumab-treated GBM patients, characterized by distant infiltration of the brain by tumors that demonstrate 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 dose enhancement ratios currently reported are well within the level of radiosensitization seen in models of glioma. Zheng *et al.* used siRNA silencing of TNF receptor-associated Factor 2 (TRAF2) to radiosensitize U251 glioma cells *in vitro* with a DER of 1.2-1.39 (49). Golding

et al. reported radiosensitization with DERs of 1.6-2.1 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 et al. demonstrated 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 standard of care, radiosensitization of this magnitude reported here must be considered significant, particularly since 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 thought that the GIC population contributes to the inevitable recurrence of GBM (54). Several recent studies have shown that the TGF β pathway is important GIC biology (17-19). While we did not observe inhibition of neurosphere self-renewal with TGF β inhibition alone (seen in several of the above mentioned studies), we did find 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 multi-faceted benefits that could prevent GBM recurrence.

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Abbreviations

TGFβ	transforming growth factor β
GBM	glioblastoma
GIC	glioma-initiating cell
IR	ionizing radiation
DER	dose enhancement ratio
LAP	latency-associated peptide
ATM	ataxia telangiectasia mutated
PI3-kinase	phosphatidylinositol 3-kinase
DAPI	4',6-diamidino-2-phenylindole

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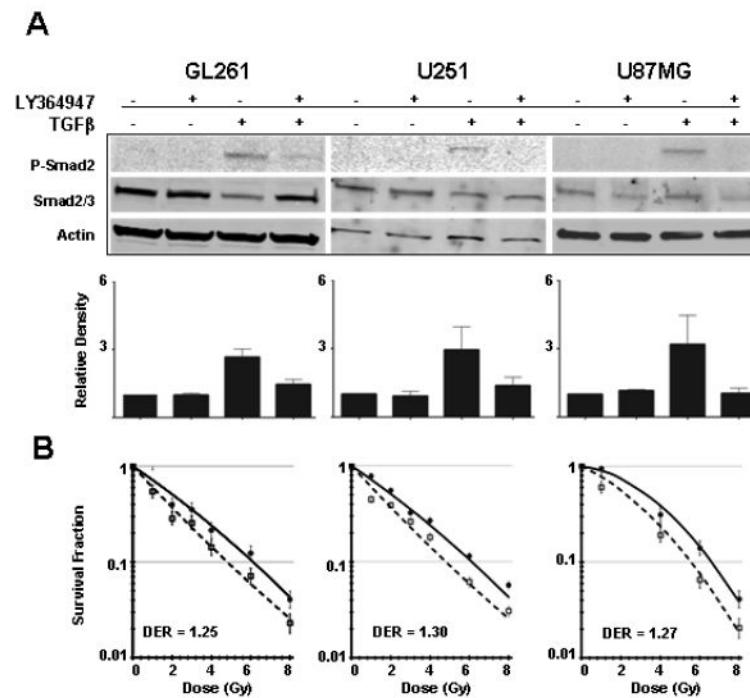


Figure 1. TGF β inhibition radiosensitizes glioma cells independent of effects on proliferation
(A) Human (U251 and U87MG) and mouse (GL261) glioma cell lines respond to exogenous TGF β 1 (2 ng/mL), as measured by Smad phosphorylation, an effect that was blocked with the addition of the TGF β type 1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

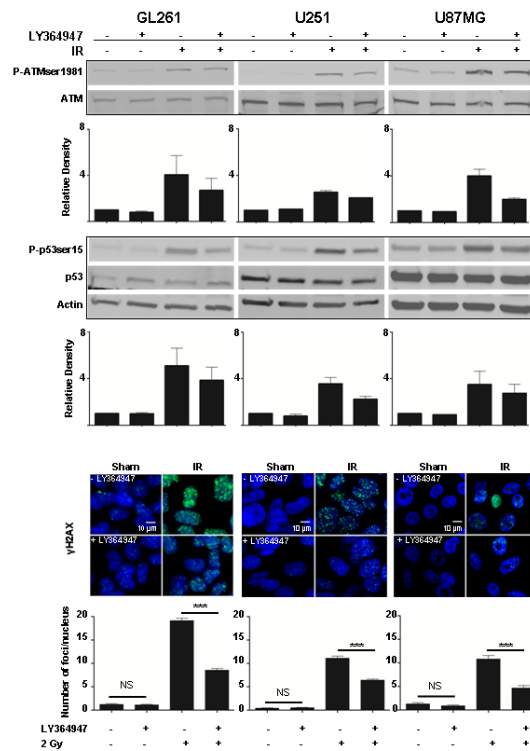


Figure 2. Radiosensitization of glioma cells by TGF β inhibition correlates with abrogation of the DNA damage response

(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. Pre-treatment 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 2-3 replicates and bar plots of densitometry quantitation of mean + S.E. are shown. (B) TGF β inhibition with LY364947 significantly decreased γ H2AX foci (green) in murine GL261 and human U251 and U87 MG glioma cells. Nuclei are counterstained with DAPI (blue). Quantification of γ H2AX foci shown below each panel revealed a significant reduction in the number of radiation-induced γ H2AX foci with LY364947 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.

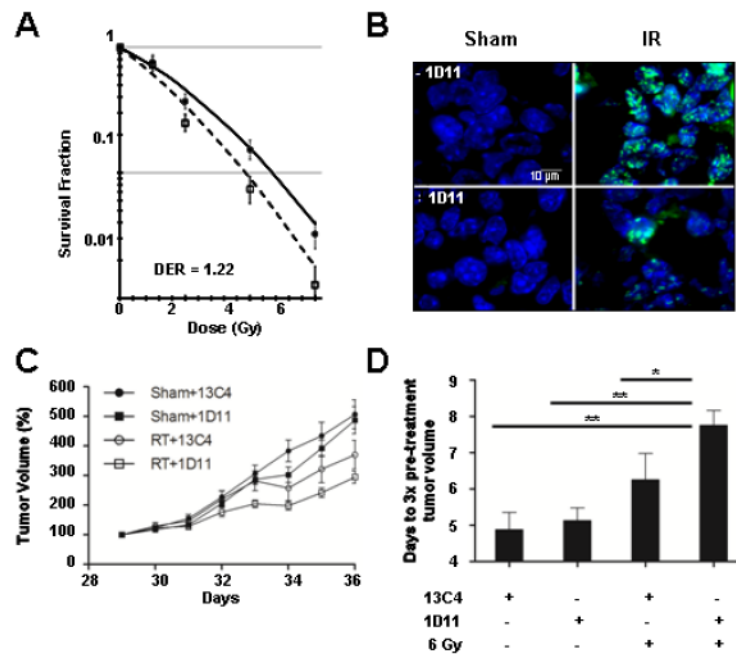


Figure 3. Treatment with 1D11 pan-specific TGF β monoclonal antibody radiosensitizes glioma cells *in vitro* and *in vivo*

(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-test. (B) Treatment with 1D11 neutralizing antibody resulted in decreased γ H2AX immunofluorescence (green) 1 hr after 2 Gy compared to irradiated mice receiving control antibody 13C4. Nuclei are counterstained with DAPI (blue). (C) A single intraperitoneal injection of 1D11 antibody 24 hours prior to irradiation (6 Gy) of GL261 flank tumors resulted in greater tumor growth delay compared to mice receiving 13C4 control antibody. The y-axis represents tumor volume normalized to pre-treatment volume. RT/13C4 vs RT/1D11, $p < 0.05$; RT/1D11 vs Sham/1D11, $p < 0.01$; ANOVA with Newman-Keuls multiple comparison post-test (D) The time-to-reach 3 times pre-treatment volume was significantly increased by 1D11 treatment compared to antibody control 13C4. * $p < 0.05$ and ** $p < 0.01$, ANOVA with Newman-Keuls multiple comparison post-test.

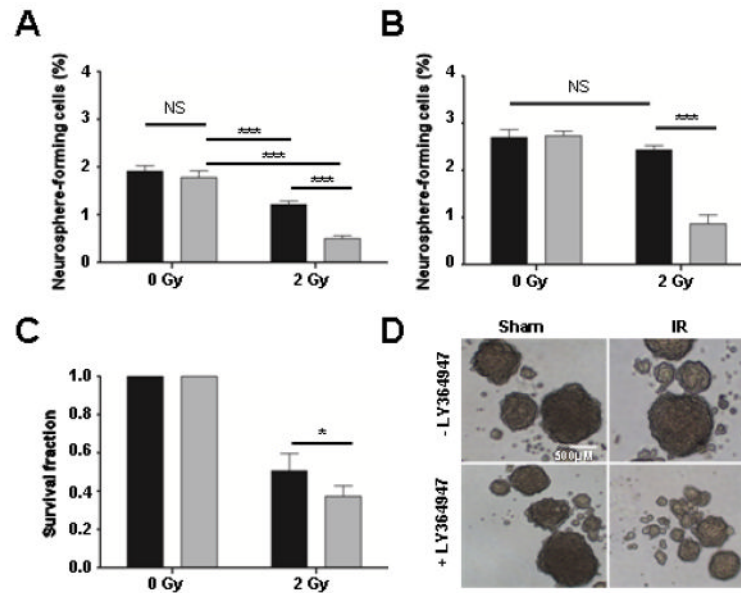


Figure 4. TGF β inhibition in conjunction with radiation decreases GL261 neurosphere-forming capacity and radiosensitizes neurosphere-derived cells

GL261 murine glioma cells were cultured under neurosphere conditions. **(A)** Treatment with LY364947 (24hrs, 400nM) alone had no effect on primary neurosphere formation.

Irradiation (2Gy) decreased primary neurosphere-forming capacity by 28%, and LY364947 treatment for 24 hours prior to irradiation decreased neurosphere formation by an additional 47%, resulting in 75% fewer neurospheres. **(B)** Treatment with LY364947 (24hrs, 400nM) 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)** Pre-treatment with LY364947 for 24 hr decreased neurosphere-derived clonogenic cell survival after irradiation (2Gy). After 2 Gy irradiation the survival fraction of untreated neurospheres was reduced by 43% while the survival fraction of neurospheres treated with 2Gy and LY354947 was further reduced by an additional 20%.

(D) Representative images of neurospheres from sham, control-treated, sham, LY364947 -treated, 2Gy, control-treated, and 2Gy, LY364947 -treated. Data are means+S.D. of triplicate determinations and representative three experiments. NS, not significant, * $p < 0.05$ and *** $p < 0.0001$, ANOVA.

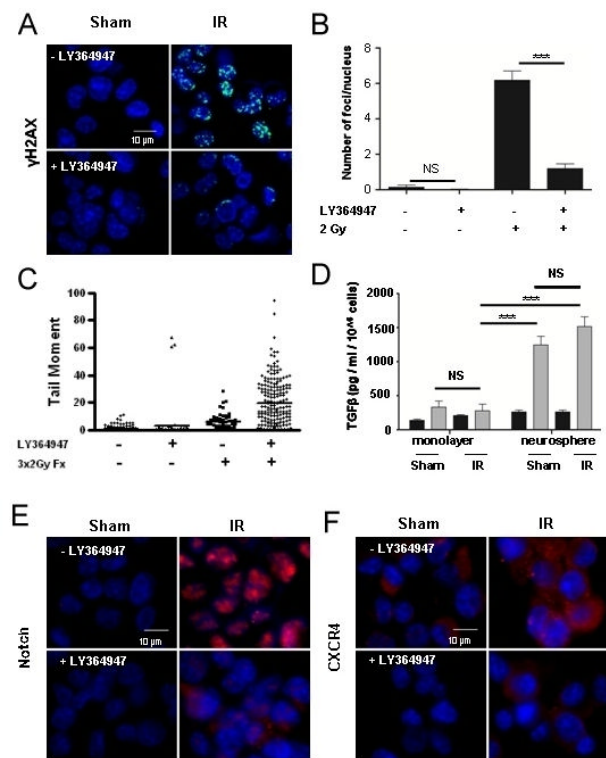


Figure 5. TGF β inhibition affects DNA damage response and self-renewal pathways up-regulated by ionizing radiation

(A) LY364947 pre-treatment for 24 hours prior to 2 Gy decreased radiation-induced γ H2AX foci in GL261 cells derived from neurosphere. (B) Quantification revealed an 81% decrease in the number of radiation-induced γ H2AX foci with LY364947 treatment (6.2 \pm 0.5 vs 1.2 \pm 0.3). (C) Comet assay was used to determine that LY364947 inhibited DNA repair following fractionated (3 \times 2 Gy) daily radiation exposure of neurosphere cultures. Data shown are representative of three experiments. Comets measured for control, N= 317; LY364947 treated, N=74; fractionated 3 \times 2 Gy, N=52; LY364947 treated and fractionated 3 \times 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. *** indicates $p < 0.0001$, single-tailed unpaired t-test. (E) Inhibition of TGF β with LY364947 also blocked radiation-induced Notch1 immunofluorescence (red), and (F) CXCR4 immunofluorescence (red), in GL261 cells dissociated from neurospheres three hours after irradiation with 2 Gy. Nuclei are counterstained with DAPI (blue).