Microenvironment and Immunology

Blockade of TGF-β Signaling by the TGFβR-I Kinase Inhibitor LY2109761 Enhances Radiation Response and Prolongs Survival in Glioblastoma

Mengxian Zhang1,2,4, Susanne Kleber5, Manuel Röhrich2,4, Carmen Timke2,4, Na Han1, Jochen Tuettenberg5, Ana Martin-Villalba3, Juergen Debus4, Peter Peschke2, Ute Wirker2, Michael Lahn6, and Peter E. Huber2,4

Abstract

Glioblastoma multiforme (GBM) is a highly aggressive primary brain tumor that tends to be resistant to the ionizing radiotherapy used to treat it. Because TGF-β is a modifier of radiation responses, we conducted a preclinical study of the antitumor effects of the TGF-β receptor (TGFβR) I kinase inhibitor LY2109761 in combination with radiotherapy. LY2109761 reduced clonogenicity and increased radiosensitivity in GBM cell lines and cancer stem–like cells, augmenting the tumor growth delay produced by fractionated radiotherapy in a supra-additive manner in vivo. In an orthotopic intracranial model, LY2109761 significantly reduced tumor growth, prolonged survival, and extended the prolongation of survival induced by radiation treatment. Histologic analyses showed that LY2109761 inhibited tumor invasion promoted by radiation, reduced tumor microvessel density, and attenuated mesenchymal transition. Microarray-based gene expression analysis revealed signaling effects of the combinatorial treatments that supported an interpretation of their basis. Together, these results show that a selective inhibitor of the TGFβR-I kinase can potentiate radiation responses in glioblastoma by coordinately increasing apoptosis and cancer stem–like cells targeting while blocking DNA damage repair, invasion, mesenchymal transition, and angiogenesis. Our findings offer a sound rationale for positioning TGFβR kinase inhibitors as radiosensitizers to improve the treatment of glioblastoma. Cancer Res; 71(23):7155–67. ©2011 AACR.

Introduction

Glioblastoma multiforme (GBM) continues to be the most frequent and most malignant human brain tumor, which is highly resistant to current conventional treatments and has one of the worst survival rates among all human cancers. New strategies to treat this deadly disease are desperately needed. Emerging evidence suggests that many cancers, including hematopoietic and solid tumors, may be driven by a small subpopulation of cancer stem–like cells [CSC; or cancer stem cells (CSC); or tumor-initiating cells (TIC)]. GBMs are among the first solid cancers in which CSCs were identified (1, 2). CSCs markers such as CD133 and side population have been used to prospectively isolate a small fraction of cells in human brain tumors with increased potential to generate tumor neurospheres and xenografts (3, 4). Recent reports suggest that the expression of the CD133 antigen in gliomas and other brain tumors could serve as a prognostic indicator for tumor recurrence, malignant progression, treatment resistance, and patient survival (5). Several studies indicate that conventional radiation therapies appear to predominantly target the better differentiated CD133+ population while leaving many CD133+ CSCs alive (6). Thus, targeting CSCs using inhibitors of TGF-β could be a promising attempt to improve glioblastoma treatment (7).

TGF-β is a family of polypeptides that regulates a wide variety of biologic functions including cell proliferation, migration, survival, angiogenesis, immunosurveillance, and embryonic stem cell maintenance and differentiation. The multifunctional effects of TGF-β are elicited through dimerization of the type I (TGFβR-I) and type II (TGFβR-II) serine/threonine kinase receptors. Upon TGF-β binding, the receptor complex phosphorylates the transcription factors SMAD2 and SMAD3, which then binds to SMAD4 and translocates to the nucleus, where they regulate transcription of various target genes (8). The overexpression of TGF-β
ligands has been reported in various malignant entities including malignant gliomas (9–11). In patients with malignant glioma, elevated levels of TGF-β have been reported to be associated with high tumor grade, advanced tumor stages, and poor patient outcome (9, 12). In addition to being implicated in invasion and intratumoral angiogenesis in glioblastoma, TGF-β signaling has also been reported to play a pivotal role in maintenance of stemness of glioma CSLCs (13, 14). These multiple roles of TGF-β in glioma initiation and progression have promoted the development of therapeutic agents based on the inhibition of the TGF-β pathway (13, 15). LY2109761, a novel TGFβR-I kinase inhibitor, has shown a SMAD2-selective inhibitory profile with antitumor activity in various tumor models such as colorectal cancer (16), pancreatic cancer (17), and hepatocellular carcinoma (18).

Because radiotherapy is a mainstay treatment modality in glioblastoma but may promote radioresistance in glioma stem cells (6), a combination with an inhibitor of TGF-β, which targets in particular CSLCs (7), seems promising. We investigated here the combination effects of radiation and LY2109761 in established human glioblastoma cell lines and in GBM CSLCs in vitro and in vivo in a subcutaneous and an orthotopic tumor model. Our data indicate that LY2109761 is an effective treatment approach alone and augments the radiation treatment response in particular in GBM CSLCs.

Materials and Methods

Cell cultures and treatment conditions

The human GBM cell lines U87MG and T98 were obtained from the American Type Culture Collection. CD133+ glioblastoma CSLCs (NMA-23) were maintained in their undifferentiated state using neurobasal media supplemented with epidermal growth factor and fibroblastic growth factor, sodium pyruvate, glutamine, B27, nonessential amino acids, and penicillin/streptomycin (Gibco). NMA-23 cells were isolated from human glioblastoma surgical sample using phycoerythrin (PE)-labeled CD133 antibody (Miltenyi Biotech) as previously described (19). The collection of human biopsy tissue was approved by the regional ethical committee. LY2109761 was kindly provided by Eli Lilly, constituted in dimethyl sulfoxide (10 mmol/L), and stored at −20 °C. Cell exposures with LY2109761 were conducted 2 hours prior to irradiation or transfected with short interfering RNA (siRNA) targeting TGFβR-I or nonsense siRNA for 48 hours were plated in 25 cm² flasks, irradiated, and returned to the incubator for 10 to 14 days. Colonies formed were stained with crystal violet (Sigma) and those with at least 50 cells were counted by microscopic inspection. The linear quadratic equation was fitted to data sets to generate survival curves, and dose enhancement factor for drugs was calculated at 10% surviving fraction (DEF0.1).

Clonogenic assay

Increasing numbers of cells [pretreated with LY2109761 for 2 hours or transfected with short interfering RNA (siRNA) targeting TGFβR-I or nonsense siRNA for 48 hours] were plated in 25 cm² flasks, irradiated, and returned to the incubator for 10 to 14 days. Colonies formed were stained with crystal violet (Sigma) and those with at least 50 cells were counted by microscopic inspection. The linear quadratic equation was fitted to data sets to generate survival curves, and dose enhancement factor for drugs was calculated at 10% surviving fraction (DEF0.1).

Neurosphere formation, limiting dilution, and proliferation assay

For the neurosphere formation assay, 1,000 NMA-23 cells (pretreated with LY2109761 for 2 hours or transfected with siRNA targeting TGFβR-I or nonsense siRNA for 48 hours) were seeded on 25 cm² flasks and irradiated. After 7 days of incubation, numbers of neurospheres consisting of at least 50 cells were counted under a microscope. For the limiting dilution assay, increasing numbers of NMA-23 cells were plated in 96-well plates in 200 μL serum-free medium and treated with radiation or LY2109761 or their combination. After 7 days of culture, the percentage of wells not containing neurospheres for each cell-plating density was calculated and plotted against the number of cells plated per well. For the proliferation assay, 10,000 NMA-23 cells were seeded on 25 cm² flasks overnight and then treated as described, incubated for another 72 hours, when the numbers of living cells were counted.

Immunofluorescent staining for γH2AX

Cells were grown and treated in chamber slides. At specified time points, cells were fixed with 3% paraformaldehyde for 10 minutes and then permeabilized with 0.5% Triton X-100 for 30 minutes on ice. Antibody [AlexaFluor 488 anti-H2AX phosphorylated (Ser139); Biolegend] was added at a dilution of 1:100 in 3% bovine serum albumin and incubated overnight at 4 °C. Cells were washed and nuclei were counterstained with 1 μg/mL 4',6-diamidino-2-phenylindole. Foci were counted by the automated image analysis system Metacube with a Zeiss Axiosplan-2 imaging epifluorescence microscope equipped with ISIS software (MetaSystems). For each treatment condition, γH2AX foci were analyzed in at least 400 cells and median number of foci was determined.

Apoptosis and flow cytometry

Twenty-four hours after treatments, U87MG and NMA-23 cells were prepared for FACS analysis (FACScan; Becton Dickinson). Cells were fixed in 70% ethanol, centrifuged, washed in PBS, and the supernatant was removed. Cells were resuspended in the staining solution of PBS, RNase (200 μg/mL), and propidium iodide (10 μg/mL) and were analyzed for cells with sub-G1 DNA content. To measure caspase-3 activity, cells were treated according to the manufacturer’s suggestions (BD Pharmingen), incubated for 20 minutes on ice, pelleted, washed, and resuspended in washing buffer plus PE-conjugated monoclonal active caspase-3 (BD Pharmingen) and analyzed by flow cytometry and FlowJo software (Tree Star, Inc.).
Western blot analysis
Six hours after treatment as indicated, cells were washed twice with PBS, and lysed using Qproteome Mammalian Protein Prep Kit (QIAGEN GmbH) supplemented with a complete protease and phosphate inhibitor cocktail (Sigma). Protein concentration was measured using a Bradford assay (Pierce Chemical Co.), and samples were immunoblotted with antibodies against phospho (p)-SMAD2 and total SMAD2 (Cell Signaling) with an anti-β-actin monoclonal antibody (Sigma) as internal loading control.

Microarray analysis
U87MG cells were solubilized and homogenized in TRIzol (Invitrogen) 6 hours after radiation with or without LY2109761 treatment (4 Gy, 10 μmol/L). For LY2109761 treatment, cells were exposed to LY2109761 for 2 hours prior to irradiation as in the other in vitro assays. Total RNA was isolated according to the manufacturer’s instruction, purity and integrity of the RNA were assessed with Agilent 2100 BioAnalyzer (Agilent Technologies), and 200 ng of total RNA was used for amplification and labeling using the Low Input Quick Amp Labeling Kit (#5190-0442) from Agilent following the detailed kit protocol. Cyanine 3–labeled cRNA was purified using RNeasy mini spin columns from QIAGEN. Quantity and cyanine incorporation of labeled cRNAs were determined using a Nanodrop ND-1000 spectrophotometer (Pqlab Biotechnologies GmbH). The labeled probes were then hybridized to an Agilent 4 × 44 K whole human genome microarray (GH112F) containing 45,015 features representing 41,000 unique probes. After washing, microarrays were scanned using an Agilent Array scanner (Agilent Technologies). Data were extracted with Agilent feature extraction software (Agilent version 9.1) and statistically analyzed using SUMO software as previously described (20). Ingenuity Pathway Analysis was used for functional analysis (IPA: Ingenuity Systems, Inc.; ref. 21). Microarray data were deposited in “ArrayExpress” (Accession No.: E-TABM-1148).

qRT-PCR
To quantify mRNA expression, qRT-PCR was carried out using Quantitect Primer assay (QIAGEN GmbH) and QuantiTect SYBR Green RT-PCR Kit (#204243; QIAGEN) on a LightCycler 480 instrument (Roche Diagnostics). The relative expression of the target genes was calculated by normalizing the C_p (crossing point) values with those of housekeeping gene GAPDH.

Tumor growth in the subcutaneous U87MG model in BALB/c mice
All animal experiments were approved by in-house and governmental animal protection committees. A total of 5 × 10^6 U87MG cells were injected subcutaneously into the right hind limb of 6- to 8-week-old BALB/c athymic nude mice (Charles River Laboratories). Animals were randomized into 4 groups (control, LY2109761, radiation, radiation plus LY2109761; n = 10 each). Treatments started when tumors were established and reached a volume of approximately 150 mm^3. Tumors were irradiated with fractionated radiotherapy (5 × 2 Gy, days 0–4 for 5 consecutive days) using a 6 MV LINAC (Siemens). LY2109761 was administered orally at 50 mg/kg twice daily (days 1–5 of each week) until the end of observation. Tumor volume for the subcutaneous experiment was determined 3 times weekly by direct measurement with calipers (volume = length × width × width × 0.5).

Tumor growth and animal survival in the orthotopic CSLC model in SCID mice
Beige severe-combined immunodeficient (SCID) mice (8-week-old, 20 g; Charles River Laboratories) were anesthetized and stereotactically inoculated with NMA-23 cells (10^4 cells in 2 μL PBS) into the left forebrain (2 mm lateral, 1 mm anterior to bregma, at 3 mm depth from skull surface). Animals were randomized into 4 treatment groups (control, LY2109761, radiation, radiation plus LY2109761; n = 13 each, with 4 mice scheduled for histology). LY2109761 treatment (50 mg/kg twice daily) started on day 1 after tumor inoculation and was administered 5 days weekly until the end of observation. Radiation was delivered on day 4 to the entire head of anesthetized mice (7 Gy single dose) using the 6 MV LINAC. On day 15, animals were examined by MRI at 1.5 T (Siemens Magnetom Vision) using a custom-made small animal solenoid Tx/Rx radiofrequency coil. Tumor volumes were estimated using gadolinium-enhanced T1-weighted spin-echo images. A 3-dimensional reconstruction of the tumor surface and calculation of the tumor volume were conducted with the QuickVol software as previously described (22). For survival studies, moribund mice or mice with severe neurologic symptoms were euthanized.

Tumor histology and immunohistochemistry
Four animals per group were sacrificed on day 16 and exsanguinated by transcardial perfusion first with ice-cold PBS. Brains were dissected, partially cryo, or embedded in paraffin after fixation in 4% paraformaldehyde for 24 hours. Sections of paraffin-embedded blocks were stained with hematoxylin and eosin. Immunohistochemistry was carried out with frozen sections as previously described (23). Primary antibodies included the following: anti-vimentin (rabbit polyclonal; Abcam; 1:200), anti-CD31 (rabbit polyclonal; BD Biosciences Pharmingen; 1:100), anti-fibronectin (rabbit polyclonal; Abcam; 1:400), anti-COL5A1 (rabbit polyclonal; Santa Cruz; 1:100), and anti-YKL-40 (rabbit polyclonal; Quidel; 1:200). After incubation with primary antibodies, the appropriate fluorescence-labeled secondary antibodies were applied. Images were captured using a Nikon Eclipse E600 microscope equipped with a Nikon digital sight DS-U1 camera and subsequently analyzed using Imagej software (NIH, Bethesda, MD). For each treatment condition, the analysis was conducted in at least 5 randomly chosen fields from 3 to 5 sections.

Statistical analysis
The unpaired 2-tailed t test was used for the comparison of parameters between groups. The Kaplan–Meier method was used to determine the median survival time (MST), and a log-rank test was used to compare the differences between survival curves. A value of P < 0.05 was considered significant. The statistical analysis was conducted using the software package Statistika 6.0 (Statsoft).
LY2109761 enhanced radiosensitivity of glioblastoma cells

To determine the effects of LY2109761 on GBM tumor cell radiosensitivity, clonogenic survival analysis was conducted. LY2109761 pretreatment reduced clonogenic survival in cell cultures of U87MG (Fig. 1A) and T98 (Fig. 1B) following radiation, resulting in an increase in the radiosensitivity with a DEF0.1 of 1.30 and 1.37, respectively. Likewise, TGβFR-I siRNA reduced TGβFR-I mRNA expression and inhibited SMAD2 phosphorylation (Supplementary Fig. S1), associated with an increase in the radiosensitivity with a DEF0.1 of 1.34 in U87MG cells (Fig. 1C), supporting the notion that LY2109761 exhibits its radiosensitizing effect via TGF-β signaling blockage.

LY2109761 reduced GBM-derived CSLC self-renewal and proliferation and sensitizes them to radiation

Considering that glioblastoma stem cells have been linked to radioresistance, we explored whether LY2109761 would inhibit the self-renewal of GBM CSLCs and increase the sensitivity of these TICs toward radiation. We used neurosphere formation as a surrogate marker of the clonogenic survival of glioblastoma CSLCs given they grow in suspension (24). We found that LY2109761 (10 μmol/L) or radiation (4 Gy) alone reduced neurosphere-forming efficiency in NMA-23 cells. The combination of LY2109761 plus radiation had supra-additive effects in neurosphere formation and limiting dilution assays (Fig. 2A, B, and D). Likewise, blockage of TGF-β signaling via TGβFR-I siRNA alone or combined with radiation also reduced clonogenicity of NMA-23 cells (Fig. 2C). Furthermore, a proliferation assay revealed a reduction of NMA-23 cells proliferation/viability after LY2109761 or radiation treatment alone, and their combination resulted in a further reduction of the cell count (Fig. 2E).

LY2109761 enhanced radiation-induced DNA damage and apoptosis rates in glioblastoma cells and GBM-derived CSLCs

DNA damage and repair are important components of radiation-induced cytotoxicity. As a marker of recognized DNA double-strand breaks (DSB), we evaluated the induction of nuclear foci of phosphorylated histone H2AX (γH2AX). U87MG and NMA-23 cells were pretreated with LY2109761 (10 μmol/L) for 1 hour and irradiated (2 Gy). In both cells, LY2109761 significantly increased the number of radiation-induced γH2AX foci at 30 minutes and 24 hours postirradiation (Fig. 3A–C), whereas LY2109761 monotherapy did not significantly alter (P > 0.5) the number of data sets.

Figure 1. The effects of LY2109761 or TGβFR1 siRNA on radiosensitivity of glioblastoma cell lines measured by clonogenic survival assay. U87MG and T98 cells were pretreated with 10 and 5 μmol/L of LY2109761, respectively (which resulted in a surviving fraction of about 80%, data not shown), 2 hours before irradiation or transfected with siRNA targeting TGβFR-I or negative nonsense siRNA for 48 hours before radiation. Colony-forming efficiency was determined 10 to 14 days later, and survival curves were generated after correction for the cytotoxicity induced by LY2109761 alone and linear quadratic equation was fitted to data sets. A, U87MG treated with or without LY2109761. B, T98 treated with or without LY2109761. C, U87MG treated with siRNA or negative control (DEF0.1 = 1.34 when siRNA vs. negative control). Points, mean; bars, SD; DEF, dose enhancement factor; LY, LY2109761.
LY2109761 and Radiation in an Orthotopic Glioblastoma Model

Figure 2. LY2109761 or TGFβR1 siRNA reduces NMA-23 self-renewal and proliferation and sensitizes them to radiation. A, representative images (<40) of neurospheres formed by NMA-23 cells treated with LY2109761 (LY; 10 μmol/L) or radiation (RT; 4 Gy) or their combination. B, the number of neurospheres per flask, compared between the control group and cells treated as indicated. C, the number of neurospheres per flask, compared between the untreated group and cells treated with negative control, siRNA, RT, or siRNA + RT. D, NMA-23 cell self-renewal capacity was evaluated in a limiting dilution assay under each treatment condition. The data are presented as the percentage of wells not containing neurospheres for each cell-plating density. E, NMA-23 cell proliferation was determined by cell count after 72 hours of exposure to each treatment. Relative numbers of cells are shown as histogram. Columns, mean; bars, SD. *P < 0.05 versus control (or negative control); **P < 0.05 versus control (or negative control) and each monotherapy; #P > 0.05 versus untreated.

γH2AX foci. These data suggested that LY2109761 increased radiation-induced DSBs and inhibited DNA damage repair.

Next, we analyzed whether LY2109761 also affected apoptosis. We found in both U87MG and NMA-23 cells that a 2-hour pretreatment with LY2109761 before irradiation increased apoptosis rates in a supra-additive manner in both sub-G1 and caspase-3 activity assays at 24 hours after radiation (Fig. 3D and E).

LY2109761 suppressed SMAD2 phosphorylation in both glioblastoma cells and GBM-derived CSLCs

Because SMAD proteins are central mediators of signals from TGF-β receptors, we evaluated the effect of LY2109761 on the phosphorylation of SMAD2 (p-SMAD2), one of their immediate downstream targets. As expected, LY2109761 (10 μmol/L for U87MG and NMA-23; 5 μmol/L for T98) alone or combination with radiation (4 Gy) effectively suppressed SMAD2 phosphorylation (Fig. 4).
LY2109761 altered gene expression and pathway analysis in glioblastoma cells

To further investigate the potential molecular basis of the interaction of LY2109761 and radiation (RT), gene expression analysis was conducted on U87MG cells using microarrays. A heatmap was generated (Fig. 5A) representing 988 transcripts comparing significantly differentially regulated genes for each treatment condition (LY, RT, LY + RT) versus untreated controls. Selected genes were found similarly regulated in NMA-23 cells as in U87MG cells, which was confirmed using qRT-PCR (Fig. 5B; Supplementary Fig. S2), including ID1, HEP1, ANGPT2, BIRC2 (downregulated by LY2109761 treatment) and RHOB, LRG1, ACTG2 (upregulated by LY2109761 treatment) as well as AKT2, ATF5, LMO2 (upregulated by radiation and reversed by LY2109761 pretreatment).
Further analysis using IPA of differentially regulated genes with at least 2-fold change (LY, 111 genes; RT, 36 genes; LY + RT, 157 genes) revealed 8 functional categories with highly affected and enriched transcripts (Fig. 5C). The associated molecular and cellular functions are related to cellular movement, cellular growth and proliferation, gene expression, cell death, cell cycle, cell-to-cell signaling and interaction, cell signaling, and molecular transport. For example, the top function for LY2109761 and radiation alone or their combination was associated with cellular movement, cell death, and cellular growth and proliferation.

Venn diagrams revealed the treatment-specific upregulated and downregulated genes (Fig. 5D) and also the commonly affected genes (Fig. 5D). A significant number of genes were commonly affected under the treatment of LY2109761 and radiation plus LY2109761, whereas considerably fewer genes were commonly affected by radiation and radiation plus LY2109761, suggesting a comprehensive impact of LY2109761 on radiation response at the gene expression level when combined with radiotherapy.

**LY2109761 enhanced radiation-induced tumor growth delay in a U87MG subcutaneous xenograft tumor model in BALB/c nude mice**

A tumor growth delay experiment was carried out using U87MG tumors growing subcutaneously in the hind leg of nude mice. As shown in Fig. 6A, the time for 5-fold increase in tumor size (from 150 mm$^3$ to 750 mm$^3$) was calculated using the tumor volumes from the individual mice in each group. The mean time for 5-fold increase in tumor size increased from 11.4 days for sham-treated mice to 17.2 days for LY2109761-treated mice and 23.9 days for irradiated mice. Radiation plus LY2109761 increased the time to 36.4 days. The tumor growth delay versus controls was 5.8 days for LY2109761 alone, 12.5 days for radiation alone, and 25.0 days for the combination, indicating a supra-additive effect between LY2109761 and radiation on growth delay of U87MG xenografts.

**LY2109761 enhanced survival in an orthotopical CSCL glioblastoma model and enhanced antitumor activity of radiation**

The effect of LY2109761 with or without radiation on intracranial CSCL (NMA-23) tumor growth was monitored by noninvasive MRI. By day 15, both LY2109761 and radiation
monotherapy markedly inhibited tumor growth by 43.4% and 54.3% versus controls (Fig. 6B and C), which was further reduced by the combination to 76.3%. The Kaplan–Meier survival curves showed that LY2109761 alone increased modestly but statistically significantly animal survival compared with controls, with a MST of 20 versus 18 days (P < 0.05, log-rank test; Fig. 6D); radiotherapy alone prolonged the MST to 22 days. Importantly, the combination treatment with LY2109761...
plus radiotherapy further increased the MST to 28 days ($P < 0.05$ vs. respective monotherapies) indicating a supra-additive effect on tumor growth and animal survival between LY2109761 and radiotherapy in the orthotopic glioblastoma model.

LY2109761 inhibited tumor invasion and reduced tumor microvessel density

Previous studies have shown that glioblastoma cells can express the intermediate neurofilament vimentin, including those tumor cells that infiltrate into normal parenchyma. To evaluate tumor invasiveness in intracranial tumor sections, hematoxylin–eosin and immunohistochemical staining for vimentin was done. As shown in Fig. 7A and B, sham-treated intracranial xenografts displayed peripheral invasion of the surrounding brain as single cells and cell clusters, frequently surrounded by numerous small satellite tumors. Interestingly, a more invasive growth pattern was observed after radiotherapy alone than controls. In contrast, tumors from mice receiving LY2109761 monotherapy or LY2109761 plus radiotherapy displayed a significantly reduced invasion growth pattern compared with the control and radiotherapy group.

Next, we investigated tumor angiogenesis as another important process of tumor growth particularly pertinent in glioblastoma using CD31 endothelial staining and microvessel density (MVD) analysis. We found that LY2109761 alone (MVD: 39.2 ± 8.4) and radiotherapy alone (47.7 ± 10.5)
LY2109761 inhibited mesenchymal change in the CSLC orthotopic glioblastoma model

A mesenchymal character has been associated with increased malignancy, treatment resistance, and poor prognosis in glioblastoma. We found here that LY2109761 inhibited the mesenchymal character in our orthotopic CSLCs model. Immunohistochemical staining showed that LY2109761 alone and in combination with radiotherapy reduced the expression of established mesenchymal marker proteins including fibronectin, COL5A1, and YKL-40 (refs. 23, 25; Fig. 7B).

Discussion

The pivotal role of TGF-β in promoting cellular processes that are important for glioblastoma progression suggests that this pathway may be a promising target for therapy. In the present study, we provide evidence that TGF-β signaling blockade by the small-molecule TGFβR-I kinase inhibitor, LY2109761, is an effective treatment approach and can furthermore augment radiation response in human glioblastoma. This conclusion is supported by several pieces of evidence. First, LY2109761 significantly enhanced radiation-induced cytotoxicity and cooperated with radiation to suppress clonogenic survival of established glioblastoma cell lines. Mechanistic studies revealed that LY2109761 may act in concert with radiation to enhance radiation-induced DNA damage and apoptosis. Second, LY2109761 also increased the sensitivity of primary GBM CSLCs derived from surgical specimens toward radiation. Third, LY2109761 enhanced radiation-induced tumor growth delay in 2 independent models: in U87MG tumors growing subcutaneously in BALB/c nu/nu mice and in orthotopic CSLC tumors growing in brains of SCID mice. In this orthotopic brain tumor model, LY2109761 increased animal survival alone and increased the radiation-induced prolongation of the animal life span.

The interesting finding that TGF-β inhibition impedes the cellular DNA damage stress response and results in increased radiosensitivity had been shown in mammary epithelial cells (26). While these authors reported a reduced γH2AX foci number following TGF-β inhibition, we found here an increase of radiation-induced γH2AX foci number that may be due to the difference in cell types or TGF-β inhibitors used.

Targeting CSLCs in human tumors including glioblastoma is an evolving concept for developing new treatment options in cancer. Stem cells have been reported to be responsible for the initiation, propagation, recurrence, and radioresistance of gliomas (6). Notably, the most effective targeted therapies may have activity against CSLCs. Encouragingly, we found that LY2109761 reduced the self-renewal and proliferation capability of GBM CSLCs and also enhanced the radiosensitivity of GBM CSLCs. More importantly, LY2109761 further increased the antitumor effects of radiation and increased animal survival when GBM CSLCs were injected orthotopically into mice brains.

Invasion is another important feature of human glioblastoma responsible for their dismal outcomes. Although ionizing radiation is the mainstay of nonsurgical treatment in GBM, radiation may also promote migration and invasiveness of glioma cells (27), which has been attributed, in part, to reduced MVD versus controls (62.2 ± 10.55, P < 0.05; Fig. 7B).

More importantly, LY2109761 plus radiation resulted in a further reduction in MVD (23.4 ± 5.9) versus monotherapies (P < 0.05), suggesting a beneficial combination effect on reducing tumor angiogenesis in the orthotopic CSLC model (Fig. 7B and C).
increased levels of TGF-β (28). Accordingly, our results indicated that radiation may promote glioblastoma cell invasion, whereas the blockade of TGF-β signaling using LY2109761 inhibited both constitutive and radiation-provoked tumor cell invasion in the orthotopic model.

Activated tumor angiogenesis is another characteristic feature of GBM, contributing to tumor invasiveness and radio-resistance. The antiangiogenic effects of targeting TGF-β (29, 30) could be corroborated in our study because LY2109761 alone and in combination with radiation reduced tumor MVD, indicating that LY2109761 has direct antimigratory and anti-angiogenic properties, which can be beneficial in the context of an attenuation of unwanted side effects of radiotherapy such as certain promigratory and proangiogenic effects.

The fundamental role for mesenchymal change in promoting invasion, treatment response, and even CSC function in human carcinoma and GBM invasion is increasingly recognized (6, 31). Recent studies have established that TGF-β is a master regulator of epithelial-to-mesenchymal transition (EMT) in carcinoma (32). To our best knowledge, its relevance to mesenchymal change in GBM models has not been reported. Here, we found that the blockade of TGF-β signaling using LY2109761 markedly reduced the expression of mesenchymal markers in the orthotopic glioblastoma model. It is conceivable that this inhibitory effect on mesenchymal change of LY2109761, at least partly, contributes to its antimigratory capacity.

Considering that TGF-β is involved in multiple signaling pathways, genome-wide microarray analysis was conducted to investigate potential mechanisms of LY2109761, TGF-β signaling, and radiation interactions. The regulated genes represent functional classes involved in diverse biologic processes including cellular movement, cellular growth and proliferation, cell death, cell cycle, cell-to-cell signaling and interaction, and cell signaling. ID1 (along with other ID family members ID2, ID3, and ID4; data not shown), HEY1, ANGPT2, BIRC2 (cIAP1; inhibitor of apoptosis protein 1) were among the genes downregulated by LY2109761. ID1 has been reported to promote invasion, to mediate tumor angiogenesis by production of VEGF, to be involved in the resistance of cancer cells against cytotoxic drugs (33), and has also been implicated in CSC function (34) as well as EMT process (35). Our data are here in line with a very recent report focusing on the effects of LY2109761 on ID1 (36). Angiopoietins (ANGPT) are ligands of the endothelial cell receptor TIE2 and have crucial roles in the tumor angiogenic switch, inflammation, metastasis, and lymphangiogenesis. Increased expression of ANGPT2 and higher ANGPT2/ANGPT1 ratios in tumors correlate with poor prognosis in many cancers. Agents specifically targeting ANGPT1 and ANGPT2 are currently in phase II clinical trials (37). High expression levels of IAPs that inhibit apoptosis have been associated with poor treatment response and prognosis (38).

In addition, we also identified and confirmed several genes upregulated by LY2109761 treatment, including RHOB, LRIG1, and ACTG2, which act as tumor suppressors and have putative roles in tumor therapy resistance and DNA damage response (39–41).

Radiotherapy is an effective treatment modality for glioblastoma. To evade radiation toxicity, certain pathways might be activated that contribute to tumor invasiveness, angiogenesis, and radioresistance (42, 43). The set of such genes that were upregulated by radiation and reversed by LY2109761 further included AKT, ATF5, and LMO2. AKT is a major downstream effector of the phosphoinositide 3-kinase (PI3K) pathway, a potent prosurvival pathway, associated with radioresistance of cancer cells with AKT inhibition having been shown to increase radiation effects on tumors (44). Activating transcription factor 5 (ATF5) is highly expressed in malignant glioma and plays an important role in promoting cell survival by stimulating transcription of MCL1, an antiapoptotic BCL2 family member (45). ATF5 has been shown to be a potent repressor of p53, and elevated expression of ATF5 in tumor is related to radioresistance and greater cell motility (46). The LIM-domain protein LMO2 is a specific regulator of tumor angiogenesis, which has been suggested an attractive drug target in cancer (47). Together these array data may provide additional molecular signaling hints for the beneficial effects observed after combined TGF-β signaling inhibition with LY2109761 and radiation in the treatment of glioblastoma.

In summary, we have provided evidence that LY2109761, a specific TGFβR-I kinase inhibitor, is active against glioblastoma alone and enhances the antimalignant efficacy of radiation both in vitro and in vivo, in particular in GBM CSCs. Our findings rationalize further translational studies of LY2109761 or agents with similar properties alone and in combination with radiotherapy in the treatment of glioblastoma.

Disclosure of Potential Conflicts of Interest

Michael Lahn is an employee of Lilly, Inc.

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Blockade of TGF-β Signaling by the TGFβR-I Kinase Inhibitor LY2109761 Enhances Radiation Response and Prolongs Survival in Glioblastoma

Mengxian Zhang, Susanne Kleber, Manuel Röhrich, et al.

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