Targeting multiple pathways in gliomas with stem cell and viral delivered S-TRAIL and Temozolomide

Shawn Hingtgen,[1,2] Xianghui Ren,[6] Ernie Terwilliger,[6] Marie Classon,[4] Ralph Weissleder,[1,2,5] and Khalid Shah[1,2,3]

[1]Molecular Neurotherapy and Imaging Laboratory, [2]Center for Molecular Imaging Research, Department of Radiology, [3]Department of Neurology, [4]MGH Cancer Center, [5]Department of System Biology, Center for Systems Biology Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, [6]Division of Experimental Medicine, Harvard Institutes of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively kills tumor cells. However, its short half-life, poor delivery, and TRAIL-resistant tumor cells have diminished its clinical efficacy. In this study, we explored whether novel delivery methods will represent new and effective ways to treat gliomas and if adjuvant therapy with the chemotherapeutic agent temozolomide would enhance the cytotoxic properties of TRAIL in glioma lines resistant to TRAIL monotherapy. We have engineered adeno-associated virus (AAV) vectors encoding recombinant secreted TRAIL (S-TRAIL) and bioluminescent-fluorescent marker fusion proteins and show that AAV-delivered S-TRAIL leads to varying degrees of killing in multiple glioma lines, which correspond with caspase-3/7 activation. *In vivo*, dual bioluminescent imaging revealed efficient delivery of therapeutic AAV vectors directly into the tumor mass, which induced marked attenuation of tumor progression. Treatment of glioma cells with the chemotherapeutic agent temozolomide alone lead to a significant accumulation of cells in G_2-M phase, activated the cell cycle checkpoint protein Chk1, and increased death receptor expression in a time-dependent manner. Furthermore, combined treatment with AAV-S-TRAIL or neural stem cell-S-TRAIL and temozolomide induced cell killing and markedly up-regulated proapoptotic proteins in glioma cells least sensitive to TRAIL. This study elucidates novel means of delivering S-TRAIL to gliomas and suggests combination of clinically relevant temozolomide and S-TRAIL may represent a new therapeutic option with increased potency for glioblastoma patients. [Mol Cancer Ther 2008;7(11):3575–85]

Introduction

Glioblastoma multiforme is the most common brain tumor comprising 23% of all primary adult brain tumors and is the most deadly with a life expectancy of 9 to 12 months (1). Despite extensive investigation, glioblastoma multiforme remains resistant to all current therapies, with resection, radiotherapy, and chemotherapy minimally prolonging survival and ultimate redevelopment of tumors leading to patient mortality (2). Recently, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has arisen as a promising new antiglioma therapy due to its ability to induce apoptosis in a wide variety of malignant cells with high tolerance and minimal toxicity to normal tissue (3–7). Purified TRAIL protein has been shown to induce apoptosis in several different glioma lines *in vitro* (8–10), whereas both local and systemic injection of TRAIL protein exerts antitumor effects on intracranial human malignant glioma xenografts in mice (11). Despite initial clinical excitement, the rapid clearance of soluble TRAIL following systemic administration and large dose required to achieve glioma regression have limited the effectiveness of TRAIL in patients (12–14). Additionally, numerous studies have shown that a large percentage of primary glioma lines are resistant to TRAIL-induced apoptosis (15, 16). Therefore, methods to improve both efficiency of delivery and tumoricidal activity of TRAIL are required.

Two major strategies that have been used to overcome the problems of delivery encountered with recombinant proteins are viral gene therapy and cell-based therapy. Viral vectors are advantageous in that viral gene therapy can deliver robust and long-term expression of therapeutic proteins with high specificity in local tissue, reducing the systemic dose and nonspecific toxicity (17). In particular, adeno-associated virus (AAV) has received much attention in both clinical and nonclinical research due to the ability of AAV to induce high levels of transgene expression in glioma cells with minimal associated toxicity or host immune response (18–21). Alternatively, neural stem cells (NSC) have arisen as new therapeutic agents for the treatment of diseases of the central nervous system due to their unique tumor-specific homing properties, potential to differentiate into different neural cell types, and incorporate into the cytoarchitecture of the brain following transplantation (3, 22–24). Capitalizing on these properties, we and others have shown the feasibility of engineering therapeutic NSC that can efficiently delivery antitumor

Received 1/30/08; revised 8/15/08; accepted 9/3/08.

Grant support: NIH grant T32 CA79443 (R. Weissleder) and American Brain Tumor Association, Goldhirsh Foundation, Catherine and Pappas award in Neuro-oncology, and American Cancer Gene Therapy (ACGT) (K. Shah).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Khalid Shah, Massachusetts General Hospital, Harvard Medical School, Room 5421, Building 149, 13th Street, Charlestown, MA 02129. E-mail: kshah@helix.mgh.harvard.edu

doi:10.1158/1535-7163.MCT-08-0640

proteins and result in tumor regression (3, 25–27). To improve the efficacy of TRAIL, we also created a novel form of TRAIL consisting of the extracellular domain of Flt3 ligand fused to the NH_2 terminus of the extracellular domain of secreted TRAIL (S-TRAIL; ref. 28) and showed that S-TRAIL had potent bystander effects on neighboring glioma cells as well as increased cell-killing effects (4, 28). Engineering NSC with S-TRAIL (NSC-S-TRAIL), we further showed that NSC selectively migrate to established gliomas where delivery of S-TRAIL by NSC inhibited progression of human glioma xenografts assessed by serial dual bioluminescent imaging (3, 23, 27).

In an effort to improve the efficacy of TRAIL therapy and overcome TRAIL resistance, TRAIL-based combination therapies have also been investigated (7, 29, 30). Temozolomide is an oral alkylating agent used extensively in clinics as part of the chemotherapeutic regimen for treatment of high-grade glioma (31, 32). Temozolomide is a small molecule that effectively traverses the blood-brain barrier where it induces glioma cell death by causing accumulations of DNA mismatch, subsequent growth arrest, and eventually apoptosis (32, 33). Recently, studies using bacterially expressed TRAIL have highlighted a potential synergy between temozolomide and TRAIL, showing combined bacterially expressed TRAIL and temozolomide treatment had improved antitumor effects on cultured glioma cells (34). Further, convection-enhanced delivery of TRAIL protein combined with systemic administration of temozolomide proved more effective than either therapy alone in mouse models (35). These studies suggest temozolomide may sensitize cells to TRAIL-induced apoptosis; however, the underlying signaling mechanisms remain poorly understood and it is unclear whether temozolomide can enhance the killing effects of recombinant S-TRAIL delivered by novel and highly efficient AAV or NSC in glioma lines that are resistant to S-TRAIL monotherapy.

In this study, we (*a*) developed novel AAV encoding S-TRAIL and fusions between fluorescent and bioluminescent marker genes and investigated the antiglioma properties of AAV-S-TRAIL *in vitro* using different human glioma lines and *in vivo* in human glioma xenograft models, (*b*) evaluated the effects of temozolomide on glioma cells with varying resistance to TRAIL, and (*c*) evaluated the synergistic effect of therapeutic AAV-S-TRAIL and the previously developed NSC-S-TRAIL and temozolomide in human glioma cell lines with varying degrees of TRAIL resistance.

Materials and Methods

Viral Vector Generation

The recombinant Gaussia luciferase (Gluc)-DsRed2 and S-TRAIL were constructed in the AAV-MCS6 plasmid that was kindly provided by the Harvard Gene Therapy Initiative (Harvard Institute of Medicine). To first generate the Gluc and DsRed2 fusion, a 569-bp Gluc fragment was obtained from pGluc-Basic Vector (New England Biolabs) by PCR using the forward primer 5′-CTAGCTAGCGTCGACATGGGAGTCAAAGTTCTGTTTGCC-3′ bearing *Nhe*I and *Sal*I restriction sites and the reverse primer 5′-CCGCTCGAGGATATCGTCACCACCGGCCCCCTTGAT-3′ bearing *Eco*RV and *Xho*I restriction sites. The 684-bp DsRed2 fragment was similarly generated by PCR from the vector LV-firefly luciferase (Fluc)-DsRed2 (36) using the forward primer 5′-CCGATATCATGGCCTCCTCCGAGAACGTC-3′ bearing an *Eco*RV restriction site and the reverse primer 5′-CCGCTCGAGGCGGCCGCCTACAGGAACAGGTGGTGGCG-3′ bearing *Not*I and *Xho*I restriction sites. The two fragments were ligated into *Sal*I/*Not*I digested pIRES vector (Clontech Laboratories). To generate Gluc-DsRed2, a 1,898-bp fragment containing the IRES element and Gluc-DsRed2 was excised from the pIRES vector by digestion with *Eco*RI/*Not*I and ligated into *Eco*RI/*Not*I digested AAV-MCS6 vector. To generate AAV-S-TRAIL, the 1,200-bp fragment encoding S-TRAIL was obtained from LV-S-TRAIL (23) by PCR using a forward primer 5′-CGGAATTCATGACAGTGCTGGCGCCAGCC-3′ containing an *Eco*RI restriction site and a reverse primer 5′-CGACGCGTTTAGCCAACTAAAAAGGCCCCGAA-3′ containing a *Mlu*I restriction site. This fragment was ligated into *Eco*RI/*Mlu*I digested Gluc-DsRed2 vector to obtain AAV-S-TRAIL. Packaging and purification of recombinant AAV were done as described previously (37), and typical titers were 1×10^{11} particles/mL. The generation and packaging of LV-green fluorescent protein (GFP)-Fluc has been described previously (36).

Cell Lines

Human glioma cell lines Gli36 expressing a constitutively active mutant epidermal growth factor receptor (EGFR; Gli36-EGFRvIII), U251, U87, and 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-incubated fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Previous results confirmed expression of the mutant EGFR receptor in Gli36-EGFRvIII cells was shown to have no influence on TRAIL sensitivity (8). The neural progenitor cell line C17.2 has been described previously (23). Creation and characterization of the cell line Gli36-EGFRvIII-GFP-Fluc has been described previously (36). All cells were incubated at 37°C in a humidified 5% CO_2 incubator.

In vitro Fluorescence Microscopy, Bioluminescent Imaging, and Flow Cytometry

For visualization of DsRed2, 1×10^5 293T cells were plated in 24-well plates. Twenty-hour hours later, cells were transduced with 1,000 multiplicities of infection (MOI) of either Gluc-DsRed2 or S-TRAIL, and 48 h post-transduction, DsRed2 fluorescence was visualized. To confirm a linear correlation between viral transduction and Gluc photon emission, 1×10^4 293T cells were seated for 24 h in 96-well plates. Cells were then transduced with increasing MOI of either Gluc-DsRed2 or S-TRAIL for 24 h, at which time cells were incubated with 15 μg coelenterazine for 10 min, and photon emission was measured using a cryogenically cooled high-efficiency CCD camera system (Roper Scientific). For flow cytometry, Gli36-EGFRvIII glioma cells were transduced with LV-GFP-Fluc using a

MOI of 1. Following expansion in culture, the cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences) to confirm GFP-positive cells.

S-TRAIL ELISA

To determine the concentration of S-TRAIL released by AAV-S-TRAIL transduced cells, 0.4×10^6 TRAIL-resistant 293T cells were transduced with increasing MOI of control virus (Gluc-DsRed2) or S-TRAIL. Twenty-four hours later, medium was collected and TRAIL protein concentration in the medium of transduced cells was measured using the TRAIL Immunoassay Kit (Biosource International) according to the manufacturer's protocol with wells coated with human TRAIL monoclonal antibody and soluble biotinylated TRAIL polyclonal antibody. Plates were read at 450 nm using an absorbance plate reader (Molecular Devices), and the data were analyzed by SOFTMAX.

Caspase and Viability Assays

To determine the effect of S-TRAIL on cell viability and caspase activation, Gli36-EGFRvIII, U87, or U251 glioma cells were seeded in 96-well plates (1×10^4 per well). Twenty-four hours after plating, the cells were transduced with AAV-Gluc-DsRed2 or AAV-S-TRAIL at increasing MOI (0-10,000). For viability, aggregate metabolic activity was measured using an ATP-dependent luminescent reagent (CellTiter-Glo; Promega). Caspase activity was determined with a caged, caspase-3/7-activatable DEVD-aminoluciferin (Caspase-Glo-3/7; Promega). Assays were done according to the manufacturer's instructions and plates were read in a luminometer at 5 s/well. To investigate the synergistic effects of temozolomide and S-TRAIL, Gli36-EGFRvIII or U251 cells were plated as described above. Cells were treated with medium containing increasing concentrations of temozolomide (0-1,000 μmol/L) alone or in combination with the viral vectors S-TRAIL (0-4,000 MOI) or Gluc-DsRed2 (4,000 MOI) or S-TRAIL-containing medium from NSC (0-600 ng/mL) for 18 h and cell viability was determined as described above. To measure the effects of temozolomide pretreatment on S-TRAIL/temozolomide, U251 glioma cells were plated in 96-well plates and left untreated or incubated with temozolomide (100 and 500 μmol/L) for 24 h. New medium containing fresh temozolomide and control virus AAV-GlucDsRed2 (4,000 MOI), AAV-S-TRAIL (1,000 and 4,000 MOI), or NSC-S-TRAIL (200 and 600 ng/mL) was then added to both pretreated and non-treated cells, and viability or caspase activation was determined 18 h later.

Cell Cycle Analysis

Following treatment with temozolomide (100 μmol/L; 24 and 72 h), untreated and temozolomide-treated cells (Gli36-EGFRvIII and U251) were pulsed for 1 h with bromodeoxyuridine (Amersham). The cells were trypsinized and fixed in cold ethanol and denatured in 2 mol/L HCl, 0.5% Triton X-100, which was neutralized in 0.1 mol/L borate (pH 8.5). Bromodeoxyuridine incorporation was detected by incubating cells with anti-bromodeoxyuridine antibody (Becton Dickinson) for 30 min at room temperature. The cells were washed and incubated with FITC-conjugated anti-mouse antibody (Vector Labs) for 30 min. Following a second wash, the cells were incubated with propidium iodide (10 μg/mL propidium iodide; Sigma) and RNase A (250 μg/mL; Sigma) at 4°C overnight and analyzed using a Becton Dickinson FACSort flow cytometer and CellQuest software.

Cell Implantation and *In vivo* Bioluminescent Imaging

Gli36-EGFRvIII glioma cells transduced with LV-GFP-Fluc (Gli36-EGFRvIII-GFP-Fluc) has been described previously (36). Briefly, cells were transduced with LV-GFP-Fluc in growth medium containing 12 μg/mL polybrene (Fisher Scientific), visualized for GFP to confirm transduction, and expanded. Under inhaled anesthesia (1-2% isoflurane + 2 L O_2), 6×10^6 Gli36-EGFRvIII-GFP-Fluc cells in 50 μL saline were implanted s.c. into athymic nude mice (*nu/nu*; ages 6-7 weeks; Charles River Laboratories) using a 28 gauge syringe. Two days post-implantation, 1×10^9 particles of either AAV-Gluc-DsRed2 or AAV-S-TRAIL were directly injected into the tumor using a glass Hamilton syringe. A second injection of virus was administered 2 days after the initial injection. To serially monitor changes in glioma volume, mice were imaged for Fluc activity 1, 4, 7, and 12 days after the first injection of virus. D-Luciferin (4.5 mg/animal in 150 μL saline) was administered to each mouse by i.p. injection, and photon counts were recorded 5 min after D-luciferin administration with a 5 min acquisition time. Gluc imaging to visualize viral transgene expression within the tumor mass was done on mice 10 and 12 days after injection of Gluc-DsRed2. To visualize Gluc expression, mice received i.v. injection of coelenterazine (3.3 μg/g body weight) and were imaged for Gluc activity using a 5 min acquisition time 10 days post-injection of AAV. Fluc imaging to visualize tumor volume was done as described above on day 12. Postprocessing and visualization of all images was done as described previously (23, 38).

Immunohistochemistry

Seven days post-transduction by AAV, tumors were removed, placed in 10% formalin, and embedded in paraffin. Sections (10 μm) were generated and subjected to an antigen retrieval process using a sodium citrate. Sections were stained for active caspase-3/7 staining (1:50; Calbiochem) according to standard protocols followed by processing for H&E by standard techniques.

Western Blot Analysis and Immunocytochemistry

To investigate the effects of temozolomide alone, U251 cells were treated with temozolomide (100 μmol/L) for 24 or 72 h or left untreated. To determine the effects of combined temozolomide and S-TRAIL treatment on cells, U251 cells were treated for 24 h with temozolomide (500 μmol/L), NSC-S-TRAIL (600 ng/mL), a combination of temozolomide and S-TRAIL, or temozolomide and S-TRAIL following 24 h of sensitization by temozolomide alone. For all treatment groups, proteins were isolated from harvested cells, resolved by SDS-PAGE, and immunoblotted with antibodies against cleaved poly(ADP-ribose) polymerase (Cell Signaling), DR4 (Sigma-Aldrich), Chk1 (Santa Cruz Biotechnology), phosphorylated Chk1 (Cell Signaling), or α-tubulin (Sigma-Aldrich) antibodies and

detected by chemiluminescence following incubation with horseradish peroxidase-conjugated secondary antibodies. An additional group of cells plated on coverslips was similarly treated with temozolomide or S-TRAIL, fixed, permeabilized, and incubated with a cleaved caspase-3 antibody (1:200; Cell Signaling) for 1 h at 37°C. Cells were then washed and incubated with goat anti-rabbit Alexa dye (540 nm)-conjugated secondary antibody (Molecular Probes) for 1 h, washed again, mounted, and examined by fluorescence microscopy.

Statistical Analysis

Data are expressed as mean ± SD and analyzed by either Student's *t* test or ANOVA (after Bartlett's test of homogeneity of variance) followed by the Newman-Keuls' correction for multiple comparisons. Differences were considered significant at $P < 0.05$. Synergy between S-TRAIL and temozolomide was determined by the isobologram method (39). Gli36-EGFRvIII and U251 cells were treated with temozolomide at increasing concentrations alone or in combination with NSC-S-TRAIL or AAV-S-TRAIL for 18 h, at which time cell viability was determined and EC_{50} values were calculated for each drug alone or in combination with a fixed ratio of the second drug. The calculated EC_{50} values of each drug in combination were divided by the EC_{50} of each drug alone. An isobologram was generated where the dotted line connecting the EC_{50} of each drug alone represents the predicted additive value, values below the line indicate synergy and values above the line indicate antagonistic effects.

Results

To investigate the antiglioma potential of S-TRAIL delivered by AAV and simultaneously monitor transgene expression, we developed novel bimodal recombinant AAV vectors expressing a fusion of bioluminescent protein Gluc and DsRed2 (Fig. 1A). Fluorescence microscopy of TRAIL-resistant 293T cells transduced with AAV-Gluc-DsRed2 or AAV-S-TRAIL revealed robust DsRed2 expression (Fig. 1B), whereas bioluminescent imaging of AAV-Gluc-DsRed2 or AAV-S-TRAIL revealed strong Gluc signal that correlated linearly with viral concentration (Fig. 1C). ELISA on culture medium of the cells transduced with AAV-S-TRAIL showed a dose-dependent increase in S-TRAIL secretion with 1,000 MOI leading to 155 ± 17 ng/mL S-TRAIL (Fig. 1D). These results confirm the functionality of fused fluorescent and bioluminescent markers in the bimodal AAV vectors and the secretion of S-TRAIL following transduction by AAV-S-TRAIL.

Next, we investigated the effects of AAV-S-TRAIL on cell viability and caspase activation using three human glioma cell lines known to exhibit varying degrees of TRAIL sensitivity (8). AAV-S-TRAIL transduction induced a significant decrease in cell viability in Gli36-EGFRvIII (40), with 2,000 MOI leading to a 62% reduction in viability compared with control AAV-Gluc-DsRed2 transduced cells (Fig. 2A). Importantly, a corresponding dose-dependent up-regulation in caspase activity was observed in AAV-S-TRAIL transduced Gli36-EGFRvIII cells with 2,000 MOI inducing over a 3-fold increase (Fig. 2B), whereas no change in caspase activation was observed in control transduced cells. U87 cells exhibited an intermediate level of sensitivity to AAV-S-TRAIL, with 2,000 MOI inducing a 34% reduction in viability that leads to a 1.8-fold increase in caspase activation (Fig. 2C and D). U251 cells showed the highest level of TRAIL resistance with only high MOI resulting in reduction in cell viability (24% decrease; 10,000 MOI) and caspase activation (1.3-fold increase; 10,000 MOI; Fig. 2E and F). Taken together, these results suggest that S-TRAIL produced by AAV induces apoptosis in glioma cells, which correlates directly with the activation of caspases.

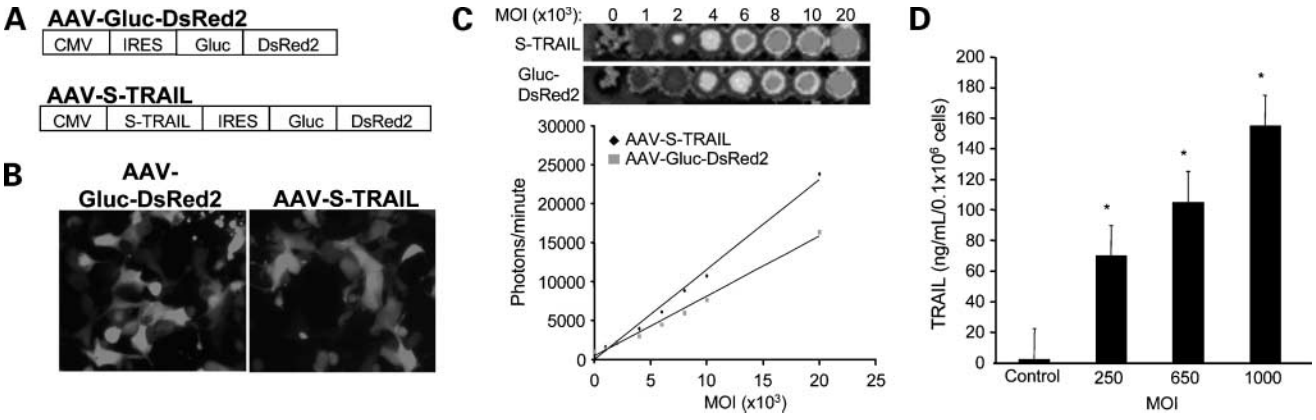

Figure 1. AAV-mediated expression of bimodal fusion proteins and S-TRAIL. **A,** schematic representation of recombinant AAV-2 vectors encoding fluorescent-bioluminescent fusion marker and S-TRAIL. **B,** TRAIL-resistant 293T cell lines were transduced with AAV-Gluc-DsRed2 or AAV-S-TRAIL (1,000 MOI), and 48 h post-infection, cells were imaged using fluorescence microscopy. **C,** representative bioluminescent images and summary graphs of 293T cell lines transduced with increasing concentrations of AAV-Gluc-DsRed2 or AAV-S-TRAIL (500-20,000 MOI). After 24 h, cells were incubated with coelenterazine and bioluminescent imaging was used to determine Gluc expression. **D,** 293T cells (4×10^5) were transduced with control (Gluc-DsRed2) or S-TRAIL, and 24 h post-infection, S-TRAIL concentration per milliliter of medium was determined by ELISA. Magnification, ×10 (**B**).

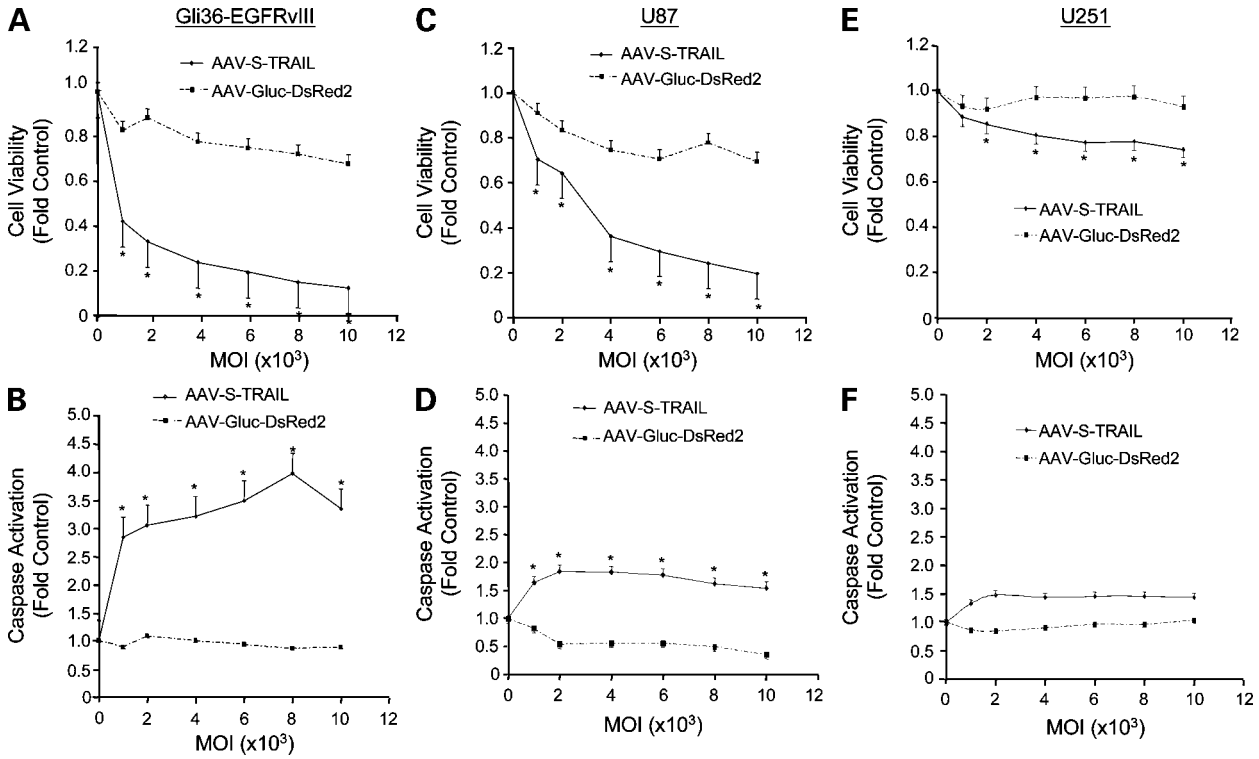

Figure 2. S-TRAIL-induced apoptosis and caspase up-regulation in human glioma cells *in vitro.* Gli36-EGFRvIII (**A** and **B**), U87 (**C** and **D**), and U251 (**E** and **F**) human glioma cells were transduced with S-TRAIL or Gluc-DsRed2 at increasing MOI (1,000-10,000), and 24 h post-transduction, both cell viability (**A, C**, and **E**) and caspase activation (**B, D**, and **F**) were determined using bioluminescent-based assays. *, $P < 0.05$ versus control.

Next, we used a glioma xenograft model to examine the effects of S-TRAIL *in vivo*. To permit noninvasive longitudinal monitoring of changes in tumor volume, we transduced Gli36-EGFRvIII human glioma cells with LV encoding GFP-Fluc fusion (Gli36-EGFRvIII-GFP-Fluc). Flow cytometry analysis showed that greater than 95% of the cells robustly expressed the GFP fusion (Fig. 3A). Representative images shown in Fig. 3B revealed that direct delivery of AAV into established Gli36-EGFRvIII-GFP-Fluc could be followed by noninvasive Gluc bioluminescent imaging. Further utilization of serial bioluminescent imaging showed that direct injection of AAV-S-TRAIL led to marked reduction in glioma burden as early as 4 days post-injection compared with control injected gliomas (Fig. 3C). This reduction in glioma volume was sustained through day 12. Histologic analysis of tumors removed 7 days post-transduction corroborated the *in vivo* findings showing significant increase in cleaved caspase-3 activation in gliomas treated with S-TRAIL compared with controls (Fig. 3D). These results show that AAV-S-TRAIL delivered directly into tumors has antitumor effects. Furthermore, bioluminescent imaging permits monitoring of both the delivery of therapeutic AAV and their efficacy in glioma models.

The results of the current study (Fig. 2), coupled with previous results from our laboratory showing human glioma lines exhibit varied degrees of sensitivity to S-TRAIL (8), emphasize the need for combination therapies to maximize the effectiveness of S-TRAIL therapy. The clinically used chemotherapeutic agent, temozolomide, has been suggested to enhance the toxicity of purified TRAIL (34). To first investigate the effects of temozolomide alone on glioma lines, cells exhibiting high (Gli36-EGFRvIII) and low (U251) sensitivity to S-TRAIL were incubated with medium containing temozolomide for 24 or 72 h or left untreated. As shown in Fig. 4A to I, temozolomide treatment of both Gli36-EGFRvIII and U251 leads to a significant inhibition in cell cycle indicated by a G_2-M arrest that was evident at 24 h and persisted through 72 h. This G_2-M accumulation was associated with a slight increase in the sub-G_1 population (Fig. 4A-I). Confirming activation of cell cycle checkpoints, Western blot analysis revealed marked increase in the levels of phosphorylated Chk1 following temozolomide treatment and no changes in total Chk1 (Fig. 4J). A marked increase in the expression of the TRAIL receptor, DR4, starting at 24 h post-treatment was observed following treatment of U251 cells with temozolomide (Fig. 4K). Alternatively, temozolomide did not increase DR4 expression above the already high levels observed in the highly TRAIL-sensitive Gli36-EGFRvIII cell line (data not shown). These results show that temozolomide inhibits cell cycle progression in glioma cells and increases the expression of death receptors, which may enhance S-TRAIL toxicity in these glioma lines.

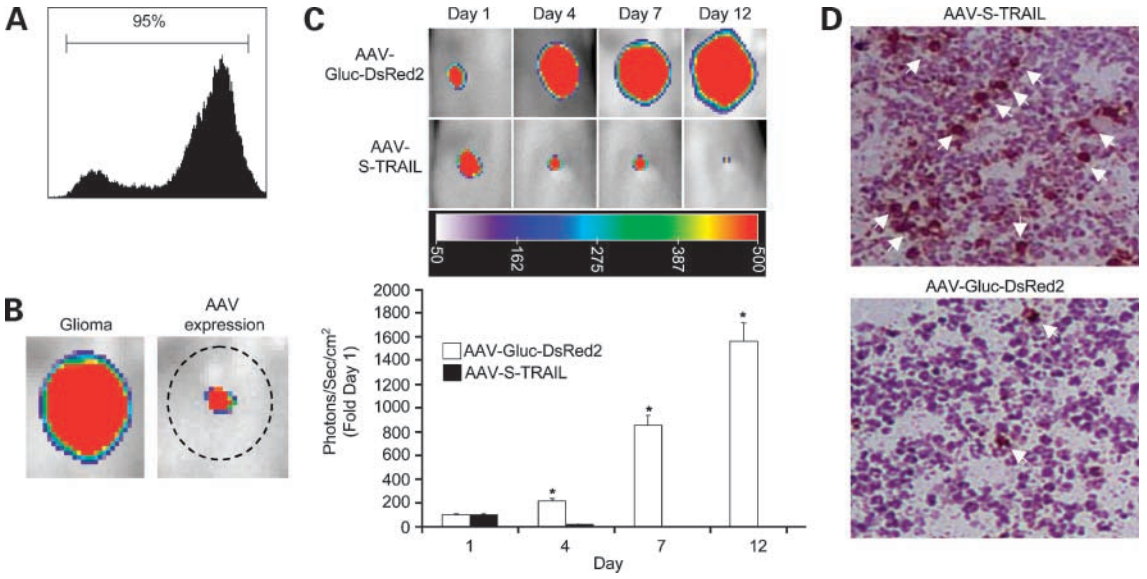

Figure 3. Real-time assessment of AAV transgene expression and effects of S-TRAIL on human glioma xenograft progression *in vivo.* **A,** flow cytometry analysis of Gli36-EGFRvIII human glioma cells transduced with LV-GFP-Fluc. Gli36-EGFRvIII glioma cells expressing GFP-Fluc were implanted s.c. into nude mice, and 48 and 96 h post-implantation, 1×10^9 particles of S-TRAIL or Gluc-DsRed2 was injected directly into the tumor mass ($n = 4$ tumors per group; day 1). Viral transgene expression and glioma progression were subsequently monitored by dual bioluminescent imaging. **B,** 10 d after delivery of AAV-Gluc-DsRed2, mice were injected with coelenterazine and imaged for viral transgene expression. Forty-eight hours later, mice were injected with D-luciferin to visualize tumor mass. Representative images are shown to show colocalization of viral expression and glioma mass. **C,** representative images and summary data showing the effects of AAV-S-TRAIL on tumor progression 1, 4, 7, and 12 d after AAV-S-TRAIL or AAV-Gluc-DsRed2 injection. **D,** 7 d post-AAV-S-TRAIL or AAV-Gluc-DsRed2 injection, tumors were removed, sectioned, and stained for caspase activation. *, $P < 0.05$ versus control.

To determine if temozolomide enhances S-TRAIL-induced cell killing, two approaches were used: (*a*) concomitant S-TRAIL and temozolomide treatment and (*b*) presensitization with temozolomide before S-TRAIL treatment. To this end, two glioma cell lines with varying degrees of TRAIL sensitivity were cotreated with AAV-S-TRAIL (0-4,000 MOI) or NSC-S-TRAIL (0-600 ng/mL) and increasing concentrations of temozolomide (0-1,000 μmol/L). Summary data shown in Fig. 5A show that cell viability in the highly TRAIL-sensitive Gli36-EGFRvIII cell line was markedly reduced by combined AAV-S-TRAIL/temozolomide treatment compared with either therapy alone. A similar reduction was observed in Gli36-EGFRvIII treated with NSC-S-TRAIL/temozolomide (Fig. 5B). In U251 cells that exhibited the highest resistance to S-TRAIL monotherapy, a modest yet significant increase in cell killing was observed in groups treated with AAV-S-TRAIL/temozolomide or NSC-S-TRAIL/temozolomide cotherapy (Fig. 5C and D).

To investigate if temozolomide pretreatment can overcome S-TRAIL resistance and to sensitize cells to combined S-TRAIL and temozolomide therapy, we first treated the minimally TRAIL sensitive U251 cells for 24 h with temozolomide alone. Following this initial sensitization, temozolomide was refreshed at which time AAV-S-TRAIL or NSC-S-TRAIL was added at low (1,000 MOI; 200 ng/mL) and high doses (4,000 MOI; 600 ng/mL). In separate groups, nonsensitized cells were treated with temozolomide and S-TRAIL simultaneously. Showing the potential effectiveness of temozolomide sensitization for overcoming S-TRAIL resistance, summary graphs shown in Fig. 6A and B show that sensitization of cells by 24 h of temozolomide pretreatment significantly enhanced cell killing of S-TRAIL/temozolomide treatment compared with cells not sensitized before S-TRAIL/temozolomide addition. In U251 cells treated with NSC-S-TRAIL, enhanced killing by temozolomide sensitization was associated with a marked increase in caspase-3/7 activation (Fig. 6C) as well as an increase in the levels of the apoptotic marker cleaved poly(ADP-ribose) polymerase (Fig. 6D) compared with nonsensitized cells. Additionally, immunocytochemistry confirmed a significant increase in the levels of cleaved caspase-3 in cells sensitized by temozolomide (Fig. 6E). These results show pretreatment of cells with temozolomide significantly increases the sensitivity of U251 glioma cells to S-TRAIL-induced apoptosis.

Discussion

Recent work in our laboratory led to the generation of a recombinant form of soluble TRAIL consisting of the extracellular domain of TRAIL fused to the extracellular domain of Flt3L by a leucine zipper (S-TRAIL; ref. 5). We have shown that S-TRAIL has significantly enhanced antitumor effects compared with TRAIL alone (5) and that the antiglioma effects of S-TRAIL produced by NSC can be enhanced through synergistic inhibition of

antiapoptotic pathways (8) or up-regulated microRNAs (23). In this study, we have engineered novel bimodal AAV vectors encoding fluorescent (DsRed2) and bioluminescent (Gluc) markers and S-TRAIL and have shown the effectiveness of AAV-S-TRAIL as monotherapy in mouse tumor models. Furthermore, we have shown that temozolomide treatment sensitizes glioma cell lines least sensitive to S-TRAIL.

In the endogenous form, TRAIL is expressed as a type II membrane-bound protein that functions as a key component of the immune system interacting with death receptors to aid in immune surveillance (41). Despite being a membrane-bound protein, the extracellular domain of TRAIL can be cleaved leading to soluble form of TRAIL that still exhibits robust tumor cell killing *in vitro* and *in vivo*. Extending this research to the clinics, Genentech and Amgen have recently initiated clinical trials to test the tumor-killing potential of soluble TRAIL in cancer patients. Current clinical trials are focused on the systemic delivery of purified TRAIL, an approach that suffers from several limitations including inefficient delivery of TRAIL to the primary glioma mass and potentially increased toxicity to normal tissue (7). Recently, other studies have focused on TRAIL delivery (3, 5, 35, 42). Previous results from our laboratory showed that therapeutic NSC-S-TRAIL represents a novel means to selectively target glioma masses, whereas the capacity to differentiate into cells of neural lineage suggests that therapeutic NSC may be able to replace neural tissue damaged by the glioma (3, 23, 27). Due to their tropism for tumor masses and ability to be engineered to secrete antitumor therapies, stem cells could be well suited for treating gliomas. However, in instances where antitumor therapies are toxic to stem cells, unable to be secreted, require a higher dose than can be delivered by stem cells, or the host rejects stem

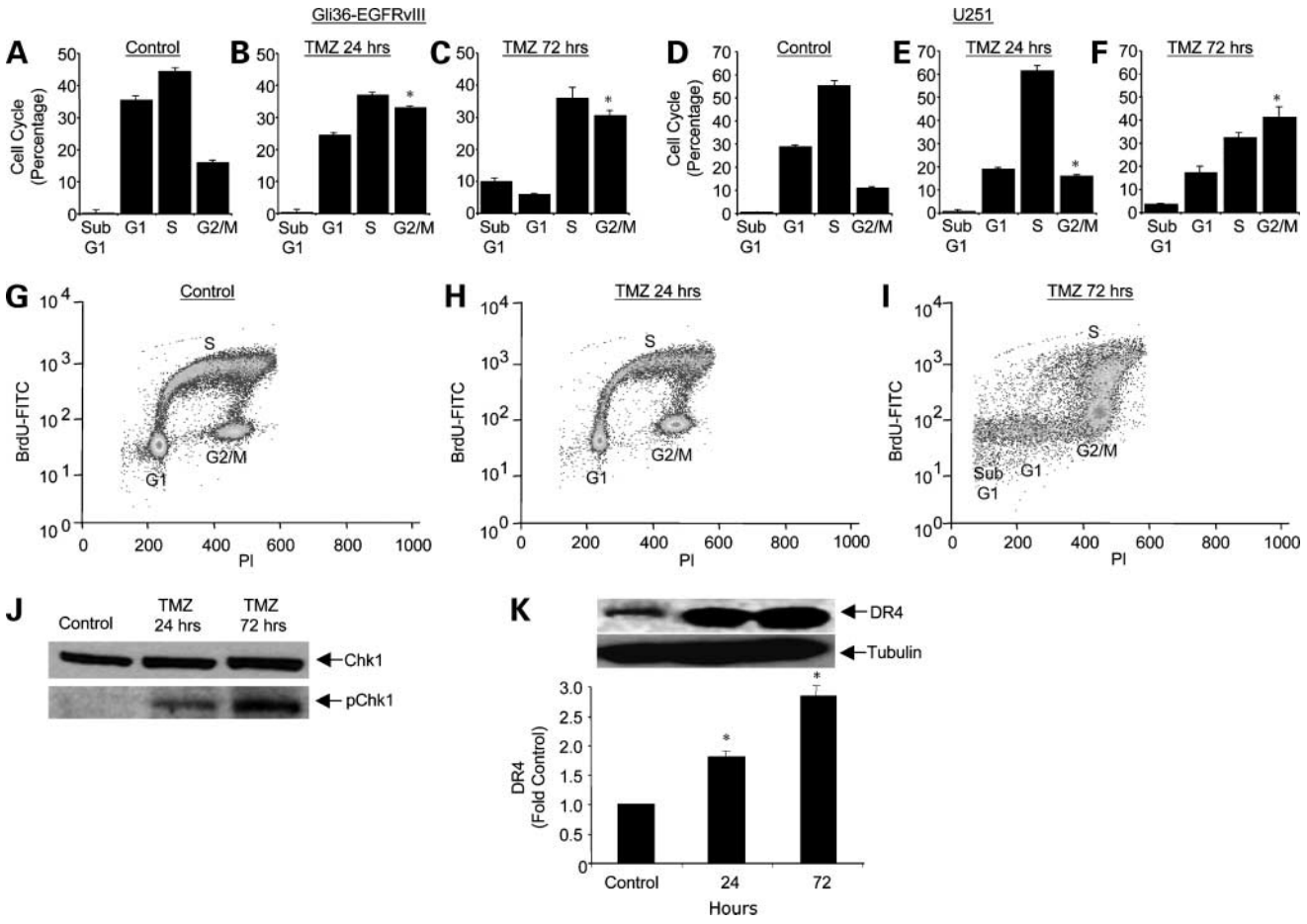

Figure 4. Temozolomide induces cell cycle checkpoint arrest and up-regulates death receptors on glioma cells. **A** to **F**, summary data of Gli36-EGFRvIII (**A-C**) and U251 (**D-F**) human glioma cells treated with temozolomide (100 μmol/L) for 24 (**B** and **E**) or 72 h (**C** and **F**) or left untreated (**A** and **D**). Cells were pulsed for 1 h with bromodeoxyuridine, collected, and stained, and cell cycle progression was analyzed by FACS. Data are expressed as percentage of cells in sub-G_1, G_1, S, or G_2-M (**G-I**). Representative scatter plots of untreated Gli36-EGFRvII cells (**G**) and cells treated for 24 (**H**) or 72 h (**I**) with temozolomide. **J**, Western blot analysis showing the effects of temozolomide on the levels of total Chk1 and phosphorylated Chk1 (pChk1). **K**, Western blot analysis showing the expression of death receptor DR4 in U251 cells treated with temozolomide for 24 or 72 h. All data are normalized to control cells (untreated). *, $P < 0.05$ versus untreated cells.

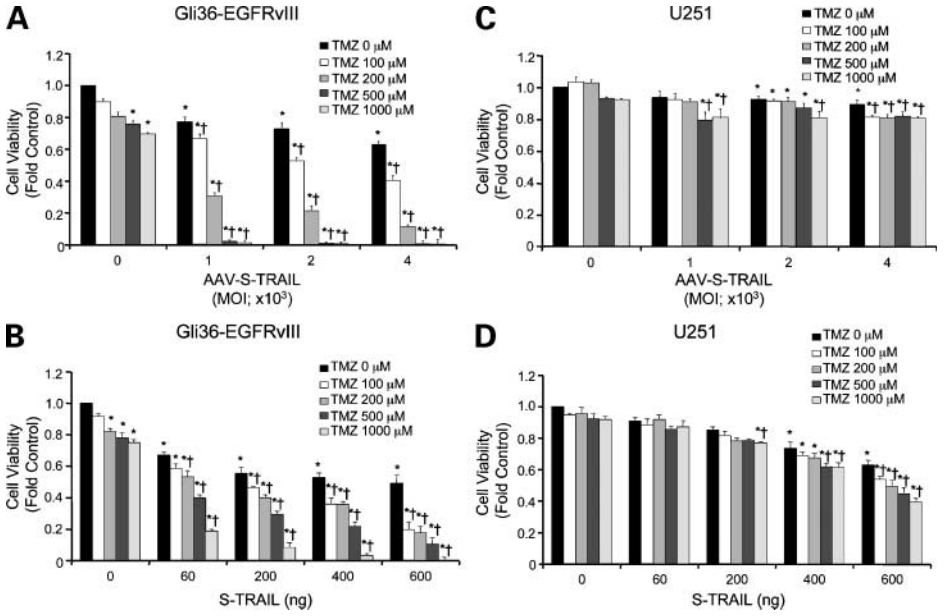

Figure 5. Synergistic cell killing by temozolomide and S-TRAIL in human glioma cell lines. Human glioma cell lines Gli36-EGFRvIII (**A** and **B**) and U251 (**C** and **D**) were treated with increasing concentrations of temozolomide alone (0-1,000 μmol/L), control virus (AAV-Gluc-DsRed2: 4,000 MOI), S-TRAIL alone (AAV-S-TRAIL: 0-4,000 MOI and NSC-S-TRAIL: 0-600 ng/mL), or a combination of temozolomide and S-TRAIL. Eighteen hours later, cell viability was determined by luciferase-based assay. All data are normalized to control cells (temozolomide: 0 μmol/L and AAV-Gluc-DsRed2 or NSC-S-TRAIL: 0 ng/mL). *, $P < 0.05$ versus control; †, $P < 0.05$ versus S-TRAIL alone.

cell engraftment, direct injection of viral vectors into tumors may prove advantageous. As such, several viral vectors have been used clinically; however, one of the most promising among these is AAV. AAV transduction occurs in both dividing and nondividing cells (20), leads to high levels of transgene expression, and can persist for several months in the brain (43). Furthermore, AAV has entered clinical trials and has been shown to have several beneficial effects over other viral vectors (reviewed in refs. 20, 28). Based on this, we developed a novel AAV-S-TRAIL as an alternative to NSC-S-TRAIL. The results of this study show that transduction of glioma cells with AAV-S-TRAIL leads to glioma cell killing *in vitro* and *in vivo* as assessed by serial bioluminescent imaging. These results suggest that the high transduction efficiency and prolonged transgene expression achieved by direct delivery of AAV-S-TRAIL offer the potential to deliver high levels of S-TRAIL therapy to localized regions of the glioma, thus minimizing toxicity to surrounding or systemic tissue. Further, the inclusion of Gluc-DsRed2 fusion proteins in this vector permits simple and noninvasive monitoring of viral gene expression that should allow easy determination of transduction efficiency and duration of expression *in vivo*, both vital criteria for achieving effective viral gene therapy. To our knowledge, this study is the first to show utility of AAV encoding TRAIL to treat malignant human glioma cells lines.

A large percentage of primary glioma lines are resistant to TRAIL-induced apoptosis (15, 16) and numerous studies have shown the potential of sensitizing TRAIL-resistant tumor cell lines by combining TRAIL with chemotherapeutic agents (8, 23, 34, 44). In this study, we show that TRAIL sensitivity can be enhanced both in lines already sensitive to TRAIL and, more importantly, in TRAIL-resistant cell lines. At concentrations as low as 100 μmol/L, temozolomide significantly increased the killing effects of S-TRAIL in Gli36-EGFRvIII. In U251 cell lines, the sensitization of cells required 24 h of pretreatment with temozolomide alone to induce TRAIL sensitivity; however, following this treatment, combined temozolomide and S-TRAIL leads to greater than 60% reduction in cell viability in this line, which showed minimal sensitivity to S-TRAIL therapy alone. The oral chemotherapy temozolomide (31), S-TRAIL (7), AAV vectors (28), and cell-based (45) therapies are all being used in the clinics. Temozolomide has been implemented as part of the standard of care for patients being treated for glioblastoma. Clinical trials with TRAIL have relied primarily on the injection of purified protein, a method that has proven inefficient. The results of this study suggest that the combination of these clinically relevant therapies may improve their delivery and potency, thus leading to improved outcomes for patients.

TRAIL-induced apoptosis is a receptor-mediated pathway, triggered on binding of TRAIL to death receptors (6, 7). Treatment of glioma cells with NSC-S-TRAIL leads to the introduction of fully functional TRAIL that can immediately activate death receptors to initiate apoptosis. In contrast, transduction of glioma cells with AAV-S-TRAIL requires cellular uptake of the virus, uncoating, integration into the cell nucleus, expression, and processing of S-TRAIL, which is then secreted (20). It is only after this secretion that we expect functional S-TRAIL to be present in the medium and capable of binding to the death receptor pathways to induce apoptosis. Therefore, we anticipate that the increased effectiveness of NSC-S-TRAIL is most likely due to this rapid death receptor binding and apoptotic induction compared with the delayed activation triggered by AAV-S-TRAIL transduction. However, both S-TRAIL

delivered by either NSC or AAV proved to have potent antiglioma effects that were synergistically enhanced by temozolomide cotherapy.

In this study, we observed temozolomide-induced accumulation of cells in G_2-M phase that was associated with up-regulation of death receptors. Several studies have clearly shown the capacity of temozolomide to induce cell cycle arrest in glioma lines. This effect is typically mediated by activation of the ATM/ATR pathways in response to DNA damage, which subsequently activates downstream cell cycle checkpoints to inhibit cell cycle progression (46–48). Various therapeutic agents have been shown to increase the expression of death receptors, including radiation (48), topoisomerase inhibitors (49), and methylating agents (49). As TRAIL toxicity is mediated by receptor binding, receptor up-regulation provides a clear link for the mechanism underlying the synergy observed between TRAIL and these antiglioma therapies. In this study, we observed temozolomide-induced up-regulation of DR4 as early as 24 h post-temozolomide treatment that remained elevated through 72 h. The increase in DR4 expression was paralleled by increased accumulation of cells in G_2-M phase and activation of Chk1. These results suggest that temozolomide and TRAIL synergism involves two mechanisms: (*a*) increase of death receptor expression in lines with low basal expression permitting increased TRAIL receptor binding and (*b*) inhibition of cell proliferation in parallel with TRAIL-induced apoptosis. As such, the first mechanism will predominate in slow proliferating cell lines with low DR4 expression (U251), whereas the second mechanism will predominate in faster proliferating cell lines with high DR4 expression (Gli36-EGFRvIII). Additional investigation will be required to elucidate the exact mechanism of temozolomide and TRAIL synergy in various cell lines; however, it is becoming increasingly clear that one of the key events underlying TRAIL/temozolomide synergy is increased expression of death receptors.

In conclusion, we have shown that antitumor effects of S-TRAIL delivered by novel means are enhanced by cotreatment with the clinical chemotherapeutic agent temozolomide. We anticipate that further investigation *in vivo* using S-TRAIL delivered by AAV or NSC and temozolomide will pave a way for their combined use in clinics. One could envision the integration of TRAIL

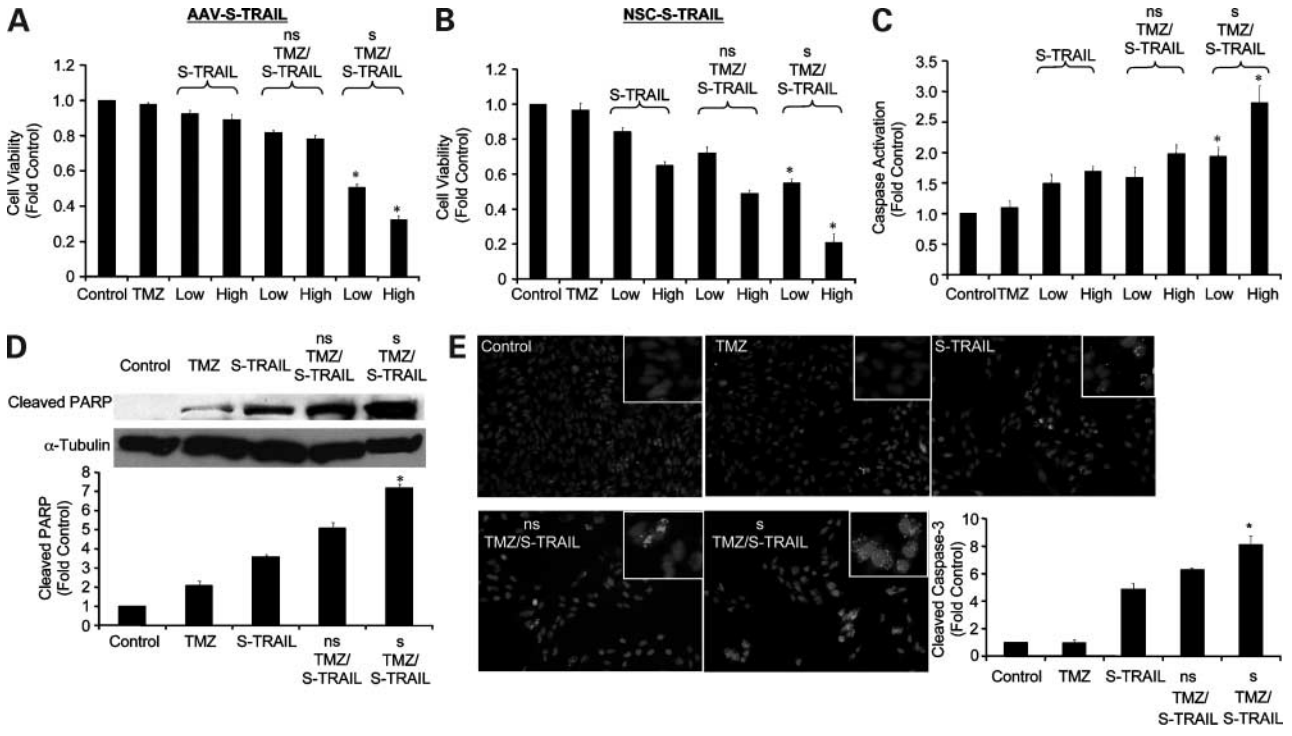

Figure 6. Temozolomide pretreatment enhances the synergistic effects of temozolomide and S-TRAIL in TRAIL-resistant glioma cells. Summary data showing the viability of U251 glioma cells following concomitant temozolomide and S-TRAIL treatment or following 24 h of temozolomide sensitization. Cells were left untreated (*ns*) or sensitized (*s*) with temozolomide for 24 h. Both sensitized and untreated cells were subsequently treated or retreated with temozolomide alone, control virus (AAV-Gluc-DsRed2: 4,000 MOI), S-TRAIL alone (Low: AAV-S-TRAIL = 1,000 MOI and NSC-S-TRAIL = 200 ng/mL; High: AAV-S-TRAIL = 4,000 MOI and NSC-S-TRAIL = 600 ng/mL), or a combination of temozolomide and S-TRAIL for an additional 18 h at which time cell viability (**A** and **B**) or caspase-3/7 activation (**C**) were determined. Representative images and summary data of Western blot analysis showing the expression of cleaved poly(ADP-ribose) polymerase (**D**) and immunocytochemical analysis of cleaved caspase-3 expression (**E**) in U251 cells following treatment with temozolomide, NSC-S-TRAIL, concomitant temozolomide and S-TRAIL, or temozolomide and S-TRAIL following 24 h of temozolomide sensitization. All data are normalized to control cells (temozolomide: 0 μmol/L and AAV-Gluc-DsRed2 or S-TRAIL: 0 ng/mL). *, $P < 0.05$ versus S-TRAIL/temozolomide no pretreatment.

therapy into temozolomide and radiotherapy currently in place as the standard of care or even eliminate the need for radiotherapy as the high level of temozolomide and TRAIL synergism should permit efficient treatment of heterogeneous gliomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Lee Zhou for providing Chk-1 antibodies and Patrick Wen, Claire Sauvageot, and Stephen Yip for expert advice on temozolomide studies.

References

1. Surawicz TS, Davis F, Freels S, Laws ER, Jr., Menck HR. Brain tumor survival: results from the National Cancer Data Base. J Neurooncol 1998; 40:151 – 60.

2. Stewart LA. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. Lancet 2002;359:1011 – 8.

3. Shah K, Bureau E, Kim DE, et al. Glioma therapy and real-time imaging of neural precursor cell migration and tumor regression. Ann Neurol 2005; 57:34 – 41.

4. Shah K, Tung CH, Breakefield XO, Weissleder R. *In vivo* imaging of S-TRAIL-mediated tumor regression and apoptosis. Mol Ther 2005;11: 926 – 31.

5. Shah K, Tung CH, Yang K, Weissleder R, Breakefield XO. Inducible release of TRAIL fusion proteins from a proapoptotic form for tumor therapy. Cancer Res 2004;64:3236 – 42.

6. Almasan A, Ashkenazi A. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine Growth Factor Rev 2003;14: 337 – 48.

7. Koschny R, Walczak H, Ganten TM. The promise of TRAIL—potential and risks of a novel anticancer therapy. J Mol Med 2007;85:923 – 35.

8. Kock N, Kasmieh R, Weissleder R, Shah K. Tumor therapy mediated by lentiviral expression of shBcl-2 and S-TRAIL. Neoplasia 2007;9: 435 – 42.

9. Jaganathan J, Petit JH, Lazio BE, Singh SK, Chin LS. Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in established and primary glioma cell lines. Neurosurg Focus 2002;13: ecp1.

10. Rieger J, Naumann U, Glaser T, Ashkenazi A, Weller M. APO2 ligand: a novel lethal weapon against malignant glioma? FEBS Lett 1998;427: 124 – 8.

11. Roth W, Isenmann S, Naumann U, et al. Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. Biochem Biophys Res Commun 1999; 265:479 – 83.

12. Kelley SK, Harris LA, Xie D, et al. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of *in vivo* efficacy, pharmacokinetics, and safety. J Pharmacol Exp Ther 2001;299:31 – 8.

13. Griffith TS, Anderson RD, Davidson BL, Williams RD, Ratliff TL. Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. J Immunol 2000; 165:2886 – 94.

14. Kagawa S, He C, Gu J, et al. Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Cancer Res 2001;61:3330 – 8.

15. Panner A, James CD, Berger MS, Pieper RO. mTOR controls FLIPS translation and TRAIL sensitivity in glioblastoma multiforme cells. Mol Cell Biol 2005;25:8809 – 23.

16. Rieger J, Frank B, Weller M, Wick W. Mechanisms of resistance of human glioma cells to Apo2 ligand/TNF-related apoptosis-inducing ligand. Cell Physiol Biochem 2007;20:23 – 34.

17. Rainov NG, Ren H. Gene therapy for human malignant brain tumors. Cancer J 2003;9:180 – 8.

18. Lawler SE, Peruzzi PP, Chiocca EA. Genetic strategies for brain tumor therapy. Cancer Gene Ther 2006;13:225 – 33.

19. Warrington KH, Jr., Herzog RW. Treatment of human disease by adeno-associated viral gene transfer. Hum Genet 2006;119:571 – 603.

20. Li C, Bowles DE, van Dyke T, Samulski RJ, Adeno-associated virus vectors: potential applications for cancer gene therapy. Cancer Gene Ther 2005;12:913 – 25.

21. Thorsen F, Afione S, Huszthy PC, et al. Adeno-associated virus (AAV) serotypes 2, 4 and 5 display similar transduction profiles and penetrate solid tumor tissue in models of human glioma. J Gene Med 2006;8:1131 – 40.

22. Shah K, Weissleder R. Molecular optical imaging: applications leading to the development of present day therapeutics. NeuroRx 2005;2: 215 – 25.

23. Corsten MF, Miranda R, Kasmieh R, Krichevsky AM, Weissleder R, Shah K. MicroRNA-21 knockdown disrupts glioma growth *in vivo* and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas. Cancer Res 2007;67:8994 – 9000.

24. Singec I, Jandial R, Crain A, Nikkhah G, Snyder EY. The leading edge of stem cell therapeutics. Annu Rev Med 2007;58:313 – 28.

25. Ehtesham M, Kabos P, Gutierrez MA, et al. Induction of glioblastoma apoptosis using neural stem cell-mediated delivery of tumor necrosis factor-related apoptosis-inducing ligand. Cancer Res 2002;62:7170 – 4.

26. Kim SK, Kim SU, Park IH, et al. Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. Clin Cancer Res 2006;12:5550 – 6.

27. Shah K. Current advances in molecular imaging of gene and cell therapy for cancer. Cancer Biol Ther 2005;4:518 – 23.

28. Rohn TA, Wagenknecht B, Roth W, et al. CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. Oncogene 2001;20:4128 – 37.

29. Cretney E, Takeda K, Smyth MJ. Cancer: novel therapeutic strategies that exploit the TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway. Int J Biochem Cell Biol 2007;39:280 – 6.

30. Stupp R, Hegi ME, Gilbert MR, Chakravarti A. Chemoradiotherapy in malignant glioma: standard of care and future directions. J Clin Oncol 2007;25:4127 – 36.

31. van den Bent MJ. Adjuvant treatment of high grade gliomas. Ann Oncol 2006;17 Suppl 10:x186 – 90.

32. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C. Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev 1997;23: 35 – 61.

33. Uzzaman M, Keller G, Germano IM. Enhanced proapoptotic effects of tumor necrosis factor-related apoptosis-inducing ligand on temozolomide-resistant glioma cells. J Neurosurg 2007;106:646 – 51.

34. Saito R, Bringas JR, Panner A, et al. Convection-enhanced delivery of tumor necrosis factor-related apoptosis-inducing ligand with systemic administration of temozolomide prolongs survival in an intracranial glioblastoma xenograft model. Cancer Res 2004;64:6858 – 62.

35. Shah K, Hingtgen SD, Kasmieh R, et al. Bimodal viral vectors and *in vivo* imaging reveal the fate of human neural stem cells in experimental glioma model. J Neurosci 2008;28:4406 – 13.

36. Xu J, Ma C, Bass C, Terwilliger EF. A combination of mutations enhances the neurotropism of AAV-2. Virology 2005;341:203 – 14.

37. Shah K, Tang Y, Breakefield X, Weissleder R. Real-time imaging of TRAIL-induced apoptosis of glioma tumors *in vivo*. Oncogene 2003;22: 6865 – 72.

38. Berenbaum MC. Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 1981;35:269 – 335.

39. Arwert E, Hingtgen S, Figueiredo JL, et al. Visualizing the dynamics of EGFR activity and antiglioma therapies *in vivo*. Cancer Res 2007;67: 7335 – 42.

40. Cretney E, Shanker A, Yagita H, Smyth MJ, Sayers TJ. TNF-related apoptosis-inducing ligand as a therapeutic agent in autoimmunity and cancer [erratum appears in Immunol Cell Biol 2006;84:238]. Immunol Cell Biol 2006;84:87 – 98.

41. Lu W, Sun Q, Wan J, She Z, Jiang XG. Cationic albumin-conjugated pegylated nanoparticles allow gene delivery into brain tumors via intravenous administration. Cancer Res 2006;66:11878 – 87.

42. Xu R, Janson CG, Mastakov M, et al. Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. Gene Ther 2001;8:1323 – 32.

43. Sayers TJ, Murphy WJ. Combining proteasome inhibition with TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) for cancer therapy. Cancer Immunol Immunother 2006;55:76 – 84.

44. Bachoud-Levi AC, Remy P, Nguyen JP, et al. Lancet 2000;356:1975 – 9.

45. Caporali S, Falcinelli S, Starace G, et al. DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. Mol Pharmacol 2004;66:478 – 91.

46. Hirose Y, Berger MS, Pieper RO. Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. Cancer Res 2001; 61:5843 – 9.

47. Guan B, Yue P, Clayman GL, Sun SY. Evidence that the death receptor DR4 is a DNA damage-inducible, p53-regulated gene. J Cell Physiol 2001;188:98 – 105.

48. Gibson SB, Oyer R, Spalding AC, Anderson SM, Johnson GL. Increased expression of death receptors 4 and 5 synergizes the apoptosis response to combined treatment with etoposide and TRAIL. Mol Cell Biol 2000;20:205 – 12.

49. Roos WP, Batista LF, Naumann SC, et al. Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O^6-methylguanine. Oncogene 2007;26:186 – 97.