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Potential Application of Temozolomide in Mesenchymal Stem Cell-Based TRAIL Gene Therapy Against Malignant Glioma

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Key Words. Mesenchymal stem cells • TRAIL • Temozolomide • Glioma

ABSTRACT

Because the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively kills tumor cells, it is one of the most promising candidates for cancer treatment. TRAIL-secreting human mesenchymal stem cells (MSC-TRAIL) provide targeted and prolonged delivery of TRAIL in glioma therapy. However, acquired resistance to TRAIL of glioma cells is a major problem to be overcome. We showed a potential therapy that used MSC-TRAIL combined with the chemotherapeutic agent temozolomide (TMZ). The antitumor effects of the combination with MSC-TRAIL and TMZ on human glioma cells were determined by using an in vitro coculture system and an in vivo experimental xenografted mouse model. Intracellular signaling events that are responsible for the TMZ-mediated sensitization to TRAIL-induced apoptosis were also evaluated. Treatment of either TRAIL-sensitive or -resistant human glioma cells with TMZ and MSC-TRAIL resulted in a significant enhancement of apoptosis compared with the administration of each agent alone. We demonstrated that TMZ effectively increased the sensitivity to TRAIL-induced apoptosis via extracellular signal-regulated kinase-mediated upregulation of the death receptor 5 and downregulation of antiapoptotic proteins, such as X-linked inhibitor of apoptosis protein and cellular FLICE-inhibitory protein. Subsequently, this combined treatment resulted in a substantial increase in caspase activation. Furthermore, in vivo survival experiments and bioluminescence imaging analyses showed that treatment using MSC-TRAIL combined with TMZ had greater therapeutic efficacy than did single-agent treatments. These results suggest that the combination of clinically relevant TMZ and MSC-TRAIL is a potential therapeutic strategy for improving the treatment of malignant gliomas.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most devastating of the brain tumors. Despite the conventional treatments of surgical resection, radiation, and chemotherapy, the median survival of GBM patients is 14.6 months for radiotherapy plus temozolomide (TMZ), 3,4-dihydro-3-methyl-4-oximidazo-[5,1-d]-1,2,3,5-tetrazine-8-carboxamide) and 12.1 months for radiotherapy alone [1]. The intrinsic heterogeneous and infiltrative nature of these tumor cells to the adjacent normal brain parenchyma, as well as their resistance to most chemotherapeutic agents available currently, are the main obstacles to the treatment of GBM [2, 3].

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor ligand family that triggers apoptosis selectively in cancer cells without damaging normal cells and tissues and is a promising candidate for cancer therapy [4–6]. Recently, a variety of stem cells that have migratory capacity toward tumors have been used as vehicles for delivering therapeutic genes to treat brain tumors [7, 8]. Because human mesenchymal stem cells (MSCs) have tumor-targeting properties, can be isolated easily, and can be engineered with viral vectors, they could have a potential clinical use in cancer gene therapy. Previous studies performed by our group and by others have shown that MSCs have a tropism for brain tumors and thus could be used as delivery vehicles for therapeutic genes [9–11], indicating that MSC-based gene therapy represents a promising strategy for improving the efficacy of the treatment of glioma. To extend the release time of TRAIL and deliver it to the infiltrated tumor cells, we evaluated MSCs as delivery vehicles for TRAIL. However, despite the expression of death-inducing TRAIL receptors, many types of cancer cells, including most glioma cells, are resistant to TRAIL-induced apoptosis [12, 13], suggesting that TRAIL alone may be an ineffective agent. Novel drugs that sensitize glioma cells to TRAIL-induced apoptosis or new strategies to overcome TRAIL resistance are needed. Recent reports
showed that chemotherapeutic agents or radiotherapy can enhance sensitivity to TRAIL by increasing the expression of TRAIL receptors in a range of tumors [14, 15], suggesting that a synergistic antitumor effect may be achieved by using combination therapies.

TMZ is an oral alkylating agent used widely in the clinical treatment of high-grade gliomas [16]. TMZ is a small lipophilic molecule that effectively crosses the blood-brain barrier and induces glioma cell death via the formation of DNA mismatches and prevention of the cycle of DNA replication [17]. Recently, TMZ has received much attention as a treatment for malignant gliomas, and a treatment of the TRAIL protein combined with TMZ was shown to be more effective than were either of the therapies alone [18, 19]; however, the underlying molecular mechanisms that TMZ can sensitize cells to TRAIL-induced apoptosis, and thus enhance the antitumor effects, remain unclear.

In this study, we demonstrated that malignant glioma cells were sensitized to TRAIL-induced apoptosis by treatment with TMZ via the upregulation of death receptors and the downregulation of anti-apoptotic proteins. Moreover, our evaluation showed that a combined therapy using TRAIL-secreting MSCs (MSC-TRAIL) and TMZ yielded more enhanced antitumor effects than did the use of the TRAIL protein with TMZ for the treatment of TRAIL-resistant gliomas. We also found that a combined therapy using MSC-TRAIL and TMZ significantly enhanced tumor regression or survival in mouse xenograft models in vivo.

**MATERIALS AND METHODS**

**Isolation and Culture of Bone Marrow-Derived MSCs**

Human bone marrow aspirates were obtained from the iliac crest of healthy donors aged 20–55 years after approval by the institutional review board of Seoul St. Mary’s Hospital (approval nos. KIRB-00344-009 and KIRB-00362-006). Bone marrow aspirate from each consented donor was collected and sent to the good manufacturing practice-compliant facility of the Catholic Institute of Cell Therapy (Seoul, Korea) for the isolation, expansion, and quality control of MSCs. The marrow mixture was centrifuged at 4°C and 793g for 7 minutes to obtain a marrow pellet. After removal of the supernatant, red blood cells were removed by adding and suspending in 10-fold volume of sterile distilled water. The cell pellet obtained by centrifuging the red blood cell-deprived sample was then cultured in Dulbecco’smodified Eagle’s medium-low glucose (PAA Laboratories, Linz, Austria, http://www.paa.at) with 20% fetal bovine serum (PAA Laboratories) at 37°C in a humidified atmosphere including 5% carbon dioxide.

**Cell Viability Assay**

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assay (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Glioma cells or MSCs (5 × 10^3) were seeded in 96-well plates to measure TRAIL-induced cytotoxicity and TMZ-mediated cell death. Cells were treated with TMZ (Sigma-Aldrich), recombinant human TRAIL protein (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), or both reagents at adequate concentrations and were analyzed 72 hours later. For coculture experiments, MSC-TRAIL were plated in Transwell inserts with 0.4-μm pores (Corning Enterprises, Corning, NY, http://www.corning.com) at various cell densities, glioma cells or MSCs were grown in the lower well of 24-well plates, and cells were analyzed 72 hours after treatment. For inhibition studies, the DRS/Fc chimeric protein (100 ng/ml; R&D Systems Inc.) was added or the caspase inhibitor Z-VAD-FMK (10 μmol/l; R&D Systems Inc.) was preincubated for 1 hour for each experiment. The plates were read using an absorbance plate reader at a wavelength of 570 nm (Molecular Devices Corporation, Sunnyvale, CA, http://www.moleculardevices.com).
Apoptosis Analysis

Apoptosis was determined using the Annexin V and propidium iodide (PI) staining-based FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). U-87MG cells (2 × 10^6 cells per well) were seeded in a six-well plate, incubated with TMZ (0–1 mM) in the absence or presence of TRAIL (100 ng/ml) for 24 hours, stained with PI and fluorescein isothiocyanate Annexin V for 15 minutes, and analyzed by flow cytometry (MoFlo; Beckman Coulter Inc., Fullerton, CA, http://www.beckmancoulter.com).

Flow Cytometry for TRAIL Receptors

Cells were analyzed for the surface expression of TRAIL receptors using phycoerythrin-conjugated anti-human death receptor 4 (DR4), death receptor 5 (DR5), and decoy receptor 1 (DcR1) and DcR2 antibodies (1 μg/10^6 cells; R&D Systems Inc.). Phycoerythrin-conjugated mouse IgG1 (1 μg/10^6 cells; R&D Systems Inc.) was used as an isotypic control. Briefly, cells (2.5 × 10^5) were stained with each antibody on ice for 30 minutes. After washing with phosphate-buffered saline (PBS), the expression levels of these death receptors were analyzed via flow cytometry.

Western Blot Analysis

Cells were lysed in 50 mM Tris-hydrochloride, pH 8.0, with 150 mM sodium chloride, 1% IGEPA L CA-630 (Nonidet P-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (RIPA) buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, http://www.roche.com). Protein extract (30 μg) was separated by SDS–polyacrylamide gel electrophoresis, and then the resolved proteins were transferred to nitrocellulose membranes (Invitrogen) and incubated with the following antibodies: goat anti-TRAIL (1:1,000; R&D Systems Inc.), goat anti-DR5 (1:500; R&D Systems Inc.), rabbit anti-Bid (1:1,000), rabbit anti-caspase 3 (1:1,000), mouse anti-caspase 8 (1:250), rabbit anti-caspase 9 (1:500), rabbit anti-Bcl-2 (1:1,000), rabbit anti-Bcl-XL (1:1,000), rabbit anti-Bax (1:500), rabbit anti-survivin (1:1,000), rabbit anti-X-linked inhibitor of apoptosis protein (XIAP; 1:500), mouse anti-cellular FLICE-inhibitory protein (c-FLIP; 1:500; Alexis Biochemical, Lausen, Switzerland, http://www.avaxoracom), rabbit anti-phospho-(p)-ERK1/2 (1:1,000), rabbit anti-ERK1/2 (1:1,000), rabbit anti-p-p38 MAPK (1:1,000), rabbit anti-p38 MAPK (1:1,000), rabbit anti-poly(ADP-ribose) polymerase (1:1,000), and mouse anti-β-actin (1:1,000; Sigma-Aldrich). All antibodies, unless otherwise stated, were purchased from Cell Signaling Technology. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; Santa Cruz Biotechnology), and the blots were detected using Amersham ECL detection reagents (GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com).

In Vitro Migration Assay

The migratory ability of MSCs was determined using Transwell inserts with 8-μm pores (Corning Enterprises), as described previously [15]. U-87MG cells (1 × 10^5) were incubated in serum-free medium for 48 hours, and the resulting conditioned medium was used as the chemotactictractant. MSCs or MSC-TRAIL (2 × 10^5 cells) were plated in Transwell inserts, and serum-free medium or conditioned medium were placed in the lower well. The filter was stained with the three-step stain set (Diff-Quik; Sysmex Corporation, Kobe, Japan, https://www.sysmex.com), and the cells that migrated to the lower side of the filter were counted under a light microscope (Axio Imager A1; Carl Zeiss, Jena, Germany, http://www.zeiss.com) for five high-power fields (×400).

The Therapeutic Effects and In Vivo Bioluminescence Imaging

All animal experiments were performed in accordance with institutional guidelines and approved by the institutional animal care and use committee of the Catholic University of Korea. Orthotopic glioblastoma xenografts were established in male athymic nude mice (6–8 weeks of age; Charles River Laboratories, Wilmington, MA, http://www.criver.com). Mice received a stereotactically guided injection of human glioma cells (1 × 10^6 U87-Luc cells in 3 μl of PBS) in the right side of the brain, as described previously [9]. Mice were divided into four groups (n = 40, with 10 per group; tumor control group, TMZ-treated group, MSC-TRAIL-treated group, and TMZ plus MSC-TRAIL-treated group). To evaluate the therapeutic effects of the combined treatment, TMZ was injected i.p. for 5 days (5 mg/kg in a mixture of saline) from 5 days after tumor inoculation. Subsequently, MSC-TRAIL (2 × 10^6 cells in 5 μl of PBS) were transplanted intratumorally at 7 days after tumor inoculation. To assess the inhibition of tumor growth via direct visualization using the Maestro 2 in vivo imaging system (CRI Inc., Woburn, MA, http://www.cri-inc.com) during the survival experiment, the substrate of luciferase, o-luciferin (150 mg of luciferin per kilogram of body weight; Xenogen, Alameda, CA, http://www.xenogen.com) was delivered via i.p. injection 10 minutes prior to bioluminescence imaging.

Immunohistochemistry

Mouse brains (n = 12, with 3 per group; tumor control group, TMZ-treated group, MSC-TRAIL-treated group, and TMZ plus MSC-TRAIL-treated group) were perfused with PBS followed by 4% paraformaldehyde under deep anesthesia. The excised brains were fixed, embedded, snap frozen in liquid nitrogen, and stored at −70°C until use. Tissues were cryosectioned (14 μm) and then stained with hematoxylin and eosin to evaluate tumor-growth inhibition. Tissues were also stained with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Roche) and developed using Cys-conjugated streptavidin (1:1,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, http://www.jacksonimmuno.com) to evaluate apoptotic activity. Some sections were stained with a cleaved caspase-3 antibody (1:250; Cell Signaling Technology) and then incubated with secondary antibody Alexa Fluor 546 goat anti-rabbit IgG (1:1,000; Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (Sigma-Aldrich).

Statistical Analysis

All data are expressed as the mean ± SEM of at least three independent experiments. Statistical differences between different test conditions were determined using the Student’s t test. A p value of <.05 was considered significant. The statistical analysis of survival was performed using the log-rank test. Synergy between TRAIL and TMZ was determined by isobologram analysis using CalcuSyn software (Biosoft, Cambridge, U.K., http://www.biosoft.com).
RESULTS

Synergistic Effect of Combined Treatment With TMZ and TRAIL

The cytotoxic effects of TMZ on human malignant glioma cells were assessed by exposing each cell line to TMZ. TMZ induced cell death in U-87MG and U-373MG cells in a dose-dependent manner but not in T98G cells, which are highly resistant to TMZ (Fig. 1A). We also showed TRAIL-induced apoptosis in human glioma cells, as determined in our previous study [15]. T98G cells were sensitive to TRAIL, U-373MG cells were resistant, and U-87MG cells had intermediate sensitivity to TRAIL. Next, we examined whether TRAIL treatment combined with TMZ enhanced the cytotoxic effect. TMZ (0.5 mM) treatment induced cell death significantly in the presence of TRAIL (100 ng/ml) in U-87MG and T98G cells (Fig. 1B), indicating that TMZ strongly sensitizes cells to TRAIL-induced cell death; however, there was only a slight cytotoxic effect in U-373MG cells, which are highly resistant to TRAIL. We also examined the effect of TMZ on TRAIL-induced apoptosis in a dose-dependent manner by Annexin V/PI-based flow cytometric analysis (Fig. 1C). The respective percentages of apoptotic U-87MG cells were 15.2% or 15.3% when treated with TMZ (1 mM) or TRAIL (100 ng/ml); however, we found that apoptosis was dramatically increased to 54% by the combination of the two (Fig. 1D). Furthermore, to assess whether the cytotoxic effects were synergistic, isobologram analysis was performed in U-87MG cells. This analysis provides a combination index value, which is a quantitative measure of the degree of drug interaction between two agents. TMZ and TRAIL act synergistically when both agents inhibit cell viability by 50% (supplemental online Fig. 1).

Effects of MSC-Delivered TRAIL Combined With TMZ

Although we showed enhanced TRAIL-induced apoptosis after combined treatment in two glioma cells, there was only a slight effect after treating U-373MG cells (Fig. 1B), which have high resistance at this concentration of TRAIL (100 ng/ml). (D): Apoptotic cell death was quantified by flow cytometry analysis and presented at each concentration relative to the value of untreated cells. *p < .05 as compared with the treatment with TRAIL alone, Student’s t test. Abbreviations: TMZ, temozolomide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Figure 1. Cytotoxic effects of combined treatment with TMZ and TRAIL on glioma cells. (A): TMZ sensitivity was determined in three glioma cells. Cells were treated with TMZ in a dose-dependent manner (0–1 mM) for 72 hours and then analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *p < .05 in the comparison of each treatment with untreated control, Student’s t test. (B): To examine the effect of combined treatment, tumor cells were seeded in 24-well plates. The cytotoxic effect on glioma cells cotreated with TMZ (0.5 mM) and TRAIL (100 ng/ml) for 72 hours was analyzed by MTT assay, *p < .05 in the comparison of each treatment with untreated control; #, p < .05 as compared with the treatment with TRAIL alone, Student’s t test. (C, D): Sensitization effect of TMZ on TRAIL-induced apoptosis in U-87MG cells was evaluated by flow cytometry analysis. (C): Apoptosis was determined using Annexin V and propidium iodide staining at 24 hours after dose-dependent treatment with TMZ (0–1 mM) in the absence or presence of TRAIL (100 ng/ml). (D): Apoptotic cell death was projected by flow cytometry analysis and presented at each concentration relative to the value of untreated cells. *p < .05 as compared with the treatment with TRAIL alone, Student’s t test. Abbreviations: TMZ, temozolomide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
Next, we examined the migratory capacity of MSC-TRAIL toward tumor cells, a unique and highly important characteristic of stem cells that renders them a very appealing therapeutic modality in MSC-based gene therapy. Results showed that genetic modification did not affect the migratory capacity of MSCs, as assessed using an in vitro migration assay (supplemental online Fig. 2C, 2D). Next, we showed that MSC-TRAIL had greater therapeutic efficacy than recombinant TRAIL by coculture experiments with U-87MG cells (Fig. 2A), and we found that coculture of MSC-TRAIL and U-373MG cells in the presence of TMZ potentiated cell death compared with treatments using a single agent (Fig. 2B). In addition, we examined the cytotoxic effects of TMZ and TRAIL on MSCs in a dose-dependent manner (0–1 mM) for 72 hours and then analyzed by MTT assay. (D): Viability of MSCs (1 x 10⁴) in response to each treatment of TMZ (0.5 mM), MSC-TRAIL (4 x 10⁴), or a combination with TMZ and MSC-TRAIL. Cells were analyzed by coculture using Transwell plates, and the viability was determined at 72 hours by MTT assay. Abbreviations: TMZ, temozolomide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MSC-TRAIL, TRAIL-secreting mesenchymal stem cells.

Figure 2. Effects of TRAIL delivered by mesenchymal stem cells (MSCs) combined with TMZ on glioma cells and MSCs. (A, B): Cytotoxic effect of MSC-TRAIL combined with TMZ (0.5 mM) on U-87MG (A) and U-373MG (B) cells was analyzed by coculture using Transwell plates, and the viability was determined at 72 hours by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. U-87MG or U-373MG cells (4 x 10⁴) were in lower wells of 24-well plates and increasing doses of MSC-TRAIL were in Transwell inserts (0.4 µm pores). *, p < .05 as compared with the treatment with MSC-TRAIL alone, Student’s t test. (C): TMZ sensitivity was determined in MSCs. Cells were treated with TMZ in a dose-dependent manner (0–1 mM) for 72 hours and then analyzed by MTT assay. (D): Viability of MSCs (1 x 10⁴) in response to each treatment of TMZ (0.5 mM), MSC-TRAIL (4 x 10⁴), or a combination with TMZ and MSC-TRAIL. Cells were analyzed by coculture using Transwell plates, and the viability was determined at 72 hours by MTT assay. Abbreviations: TMZ, temozolomide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MSC-TRAIL, TRAIL-secreting mesenchymal stem cells.

Therapeutic Potential of Combined Therapy in a Xenograft Glioma Mouse Model
We evaluated the therapeutic effect of the combined treatment in an intracranial xenograft mouse model. We established tumor-bearing mice using U87-Luc cells and monitored tumor growth using in vivo bioluminescence imaging analysis. Bioluminescence expression was decreased in mice treated with TMZ and MSC-TRAIL compared with mice treated with the control or a single agent (Fig. 3A, 3B). Furthermore, survival in tumor-bearing mice treated with MSC-TRAIL plus TMZ was significantly prolonged compared with that of the group treated with MSC-TRAIL alone (Fig. 3C). We achieved complete tumor regression or tumor-free animals after combined therapy (three survivors of seven animals) at the end of this study (until 90 days after tumor inoculation). In addition, hematoxylin and eosin staining revealed that tumor growth was more restricted, and TUNEL or cleaved caspase-3 staining showed that greater apoptosis was induced in mice treated with TMZ and MSC-TRAIL compared with the single-agent treatments (Fig. 3D). These results suggest that the combination of MSC-TRAIL with TMZ enhances therapeutic efficacy against malignant gliomas.

TMZ Sensitizes Glioma Cells to TRAIL-Induced Apoptosis via the Upregulation of DR5
Because it has been reported that the expression of death receptors is critical for TRAIL-induced apoptosis in many types of cancer, we investigated the effect of TMZ on the expression of death receptors (DR4, DR5, DcR1, and DcR2) in glioma cells. Flow cytometry analysis showed that TMZ treatment increased the cellular surface level of DR5 in U-87MG cells in a dose-dependent manner (Fig. 4A, 4B). Although previous study showed that TRAIL-induced apoptosis is preferentially mediated via DR4 in other tumor cells [23], the expression of another death receptor, DR4, was not affected by TMZ treatment in the present study. To confirm that the increase of apoptosis is mediated by DR5 upregulation, we used the DR5/Fc chimeric protein to...
block the interaction between DR5 and TRAIL. DR5/Fc efficiently blocked the apoptosis induced by cotreatment (supplemental online Fig. 3). Next, we investigated whether siRNA suppression of DR5 expression inhibited the increase in TRAIL-induced apoptosis by TMZ. Western blot analysis showed that the expression of DR5 was reduced by gene silencing and that knockdown of DR5 inhibited the increase in TRAIL-induced caspase activation compared with the treatment of control siRNA in U-87MG cells (Fig. 4C). Consistent with the Western blot analysis, cell death induced by TMZ combined with MSC-TRAIL was significantly inhibited in DR5 siRNA-treated cells compared with control siRNA-treated cells (Fig. 4D). Taken together, these results suggest that TMZ-mediated sensitization of glioma cells to TRAIL-induced apoptosis depends, at least in part, on the upregulation of DR5.

### TMZ-Induced DR5 Upregulation Is Regulated by ERK Activation

Several studies have suggested that MAPK activation plays a role in TRAIL receptor induction, thus we examined whether TMZ can activate MAPK in U-87MG cells. TMZ induced a time-dependent phosphorylation of ERK; however, TMZ had no effect on the phosphorylation of p38 MAPK (Fig. 5A). Next, to investigate whether the activation of these MAPKs is involved in TMZ-induced expression of DR5, we used siRNAs specific to ERK and p38 MAPK. Western blot analysis showed that the reduction in the expression of ERK by gene silencing correlated with suppression of the TMZ-induced upregulation of DR5 in a dose-dependent manner (Fig. 5B). By contrast, p38 inhibition had no effect on TMZ-induced DR5 expression. Next, we investigated whether siRNA suppression of ERK expression inhibited the increase in TRAIL-induced apoptosis by TMZ. Knockdown of ERK significantly inhibited the increase in TRAIL-induced poly(ADP-ribose) polymerase cleavage and caspase 3 activation compared with the treatment of control siRNA in U-87MG cells, indicating that cell death induced by TMZ combined with TRAIL was inhibited in ERK siRNA-treated cells (Fig. 5C). These results suggest that DR5 upregulation contributes to the sensitizing effect of TMZ on TRAIL-induced apoptosis and that ERK activation plays an important role in DR5 induction in glioma cells.

### TMZ Downregulates the Expression of Antiapoptotic Proteins

We examined the effect of TMZ on the expression of antiapoptotic proteins, which are important intracellular signaling proteins that...
are responsible for TRAIL-induced apoptosis. We performed Western blot analyses to detect the regulation of the antiapoptotic proteins c-FLIP, survivin, and XIAP after treatment with TMZ for 24 hours in U-87MG, U-373MG, and T98G cells (Fig. 6A). The glioma cells tested in this study exhibited TMZ-mediated downregulation of c-FLIP and XIAP in a dose-dependent manner, indicating that the suppression of c-FLIP, both the long and short isoform, and XIAP is a key event in this chemotherapy-mediated sensitization to TRAIL in line with several studies [24, 25]. Survivin was slightly downregulated in U-87MG and T98G cells; however, U-373MG cells showed slight upregulation of survivin after treatment with TMZ. In addition, the levels of other apoptosis-related proteins, Bcl-XL and Bax (data not shown), were not modulated in any of the cell lines tested. We also confirmed the level of DR5 expression after treatment with TMZ at the indicated dose in these cells. As shown in Figure 6A, TMZ upregulated DR5 expression in all glioma cell lines in a dose-dependent manner.

TMZ Enhances TRAIL-Induced Apoptosis via Caspase Activation

We performed Western blot analyses of extrinsic and intrinsic apoptotic pathways to examine whether the enhanced apoptosis observed after combined treatment is mediated by caspase activation. We measured the expression levels of caspase 8, caspase 9, caspase 3, and Bid in U-87MG cells after treatment with TMZ, TRAIL, or the combination of both (Fig. 6B). Our results showed that the expression of the active fragments of caspase 8 was enhanced after the combined treatment compared with treatment with TRAIL alone. The intrinsic pathway-related proteins (Bid and caspase 9) also became activated. Ultimately, the combined treatment resulted in a significant cleavage of caspase 3, a major effector caspase. Moreover, to determine the role of DR5 in TRAIL-induced apoptosis, we used DR5/Fc. The DR5/Fc-treated U-87MG cells exhibited inhibition of the apoptotic pathway. Next, we used the caspase inhibitor Z-VAD-FMK to confirm the role of the caspase-dependent sensitizing effect of TMZ on TRAIL-induced cell death (Fig. 6C). Pretreatment with Z-VAD-FMK significantly inhibited the death induced by the combined treatment in U-87MG cells. Taken together, these results indicate that TMZ downregulates antiapoptotic proteins, which may represent an important role of TMZ as an enhancer of TRAIL-induced apoptosis by activating multiple caspases.

DISCUSSION

Recent clinical trials of the systemic delivery of TRAIL had several limitations, including potential issues of toxicity and protein half-life, in patients treated with a high dose of TRAIL [26–28]. Because of the tropism of stem cells for tumor masses and their ability to be engineered to secrete antitumor agents, stem cells may be potent delivery vehicles for targeting gliomas. Previously, we have shown...
that MSC-based TRAIL delivery efficiently enhanced the therapeutic potential of the treatment by providing a continuous and substantial amount of TRAIL to glioma cells [9]. In this study, we demonstrated that TMZ sensitized glioma cells to TRAIL-induced apoptosis, and we provided evidence that a combined therapy using TMZ and MSC-TRAIL was a more powerful modality than treatment with a single agent against glioma, both in vitro and in vivo.

Several groups, including ours, have reported the use of MSCs expressing secretable TRAIL, which has been shown to be a potent anticancer agent in experimental glioma models [9–11, 29], and have found an effective therapeutic potential by overcoming some of the hurdles of conventional TRAIL-based treatments that use the soluble TRAIL protein or agonistic antibodies to DR5 [30, 31]. However, most glioma cells are resistant to TRAIL-induced apoptosis. Recent reports have shown that chemotherapeutic agents can sensitize TRAIL-resistant cells to the drug in a range of tumors [14, 32, 33], enhancing the antitumor effect by the combined therapy with TRAIL. In this study, TMZ effectively potentiated TRAIL-induced apoptosis in human glioma cell lines and primary glioma cells. Furthermore, we showed that MSC-based TRAIL gene therapy was more effective than treatment with the TRAIL protein, especially in the treatment of cells that are highly TRAIL resistant, which indicates the advantageous effect of the MSC-mediated delivery of TRAIL. In U-373MG cells, which exhibited the highest resistance to TRAIL-alone treatment or to combined treatment with TMZ in the present study, a significant increase in cell death was observed after treatment with MSC-TRAIL and TMZ (Fig. 2B).

Resistance to TRAIL in a variety of tumor types can be overcome by combining this drug with chemotherapeutic agents; however, TRAIL resistance may have several causes [34, 35]. Given the potentiated effects of TMZ and TRAIL observed in the present study, the identification of intracellular signaling events that are responsible for the TMZ-mediated sensitization to TRAIL-induced apoptosis is of particular importance. Although several molecular mechanisms have been implicated in TRAIL resistance, we showed that TMZ effectively potentiated TRAIL-induced apoptosis by augmenting cell death via a DR5-mediated pathway, which was verified by inhibition assays using the DR5/Fc protein to block the binding of TRAIL to the DR5 death receptor and by experiments using DR5-specific knockdown. In addition, the majority of glioma-derived cell lines are resistant to TRAIL-induced apoptosis partly because of the overexpression of c-FLIP [36]. FLIP blocks the transmission of the death signal at the level of the receptor by occupying the caspase 8 binding site on the Fas-associated protein with death domain, thus blocking the activation of the caspase cascade [37]. Other inhibitors that act downstream of the receptors have also been identified. The activation and/or activity of caspases 9, 3, and 7 can be blocked by the inhibitor of apoptosis protein family, which includes XIAP, c-IAP1, c-IAP2, and survivin [38]. Although the expression of survivin did not decrease equally, we showed that TMZ treatment caused a marked downregulation of c-FLIP and XIAP in all glioma cells tested in this study, which is in agreement with several studies verifying that these proteins had roles as critical regulators in TRAIL-induced apoptosis [24, 25]. These results suggest that the TMZ-mediated upregulation of DR5 and
downregulation of antiapoptotic proteins are key mechanisms of potentiation of TRAIL-induced apoptosis.

Although several studies have reported the antitumor effects of TRAIL, its efficacy as a single agent is insufficient [12]. Therefore, the clinical identification of safe drugs that sensitize tumor cells to TRAIL by generating synergistic effects is of great importance. In the present study, we showed that the combined therapy using TMZ and long-acting TRAIL delivered by MSCs markedly induced apoptosis in glioma cells, both in vitro and in vivo, suggesting that TMZ is a sensitizing or augmenting agent in glioma cells that are sensitive or resistant to TRAIL-induced cell death. TRAIL and TMZ have recently been investigated for the treatment of malignant brain tumors because of their low toxicity in normal tissues. In addition, our experiments showed that the mechanisms underlying these enhanced effects are related to the augmentation of caspase-mediated apoptosis via upregulation of DR5 and downregulation of antiapoptotic proteins. Understanding these mechanisms may help in optimizing TRAIL and TMZ therapy for malignant gliomas and for other cancers. In a previous report, we also showed that the combination of MSC-TRAIL with irradiation synergistically enhances the antitumor effect in the treatment of glioma [15]. Radiotherapy and chemotherapy with TMZ have been implemented as part of the standard treatment for glioma patients in the clinic. Because clinical trials of the TRAIL protein have demonstrated its inefficiency, the results of this study suggest that the combination of these clinically relevant therapies with MSC-TRAIL may improve the outcomes of patients. In addition, further investigation of the repeated administration by systemic delivery of MSC-TRAIL with controlled doses and appropriate time intervals to overcome the issues related in the relevance of limited passages and limitation to transient transfection of the TRAIL gene in MSCs will be needed for the clinical application.

Figure 6. TMZ downregulates antiapoptotic proteins and enhances TRAIL-induced apoptosis through caspase activation. (A): Western blot analysis showing expressions in DR5 and antiapoptotic proteins (c-FLIP, survivin, and XIAP) in glioma cells treated with increasing doses of TMZ (0–2 mM) for 24 hours. (B): U-87MG cells were treated with TMZ (0.5 mM), TRAIL (100 ng/ml), or a combination of TMZ and TRAIL with or without DR5/Fc chimera protein (100 ng/ml). After 24 hours, total cell extracts were analyzed by Western blot with antibodies against caspase 8, caspase 9, caspase 3, and Bid. (C): The effect of caspase inhibitor on cell death induced by TMZ plus MSC-TRAIL was evaluated. U-87MG cells (4 × 10⁴) were left untreated or treated with caspase inhibitor, Z-VAD-FMK (10 μmol/l) for 1 hour and then incubated with TMZ (0.5 mM), MSC-TRAIL (1 × 10⁴), or its combination for 72 hours, and then the cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *p < .05, in the comparison of caspase inhibitor-treated cells with untreated cells after TMZ plus MSC-TRAIL treatment, Student’s t-test. Abbreviations: Bid, BH3 interacting-domain death agonist; DR5, death receptor 5; c-FLIP, cellular FLICE-inhibitory protein (L, long isoform; S, short isoform); XIAP, X-linked inhibitor of apoptosis protein; TMZ, temozolomide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MSC-TRAIL, TRAIL-secreting mesenchymal stem cells.
CONCLUSION

This study suggests a potential application for TMZ in TRAIL-based cancer therapy. We found that TMZ sensitized glioma cells to TRAIL-induced apoptosis via modulation of the apoptotic machinery and significantly enhanced the antitumor effect of MSC-TRAIL gene therapy. Combining TRAIL-based cancer treatment with clinically relevant TMZ might be a useful strategy for improving treatment of malignant gliomas.

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AUTHOR CONTRIBUTIONS

S.M.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support; J.S.W.: collection and/or assembly of data, data analysis and interpretation; C.H.J.: collection and/or assembly of data; C.H.R.: data analysis and interpretation; J.-D. J.: provision of study material; S.-S.J.: conception and design, data analysis and interpretation, final approval of manuscript, financial support.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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