Convection-Enhanced Delivery of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand with Systemic Administration of Temozolomide Prolongs Survival in an Intracranial Glioblastoma Xenograft Model

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Abstract

Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent activator of cell death, preferentially killing neoplastic cells over normal cells, the efficacy of TRAIL for the treatment of glioma might be limited due to cellular resistance and, importantly, poor distribution after systemic administration. TRAIL and temozolomide (TMZ) were recently shown to have a synergistic antitumor effect against U87MG glioma cells in vitro. Convection-enhanced delivery (CED) can effectively distribute TRAIL protein throughout a brain tumor mass. In this study, we evaluated CED of TRAIL, alone and in conjunction with systemic TMZ administration, for antitumor efficacy. CED of TRAIL demonstrated safe and effective distribution in both normal brain and a U87MG intracranial xenograft model. Individually, both CED of TRAIL and systemic TMZ administration prolonged survival in tumor-bearing rats. However, the combination of these two treatments was significantly more effective than either treatment alone. CED of TRAIL in conjunction with systemic TMZ treatment is a promising strategy for the treatment of malignant gliomas.

Introduction

Treatment of malignant gliomas remains a challenge. The combination of surgery, radiation therapy, and chemotherapy yields a median survival of only 9 months (1). Therefore, there is a strong need for new treatment strategies. Convection-enhanced delivery (CED) is a promising local drug delivery technique. By using bulk flow, it allows the direct delivery of small and large molecules to targeted sites in clinically significant volumes of tissue, offering an improved volume of distribution over simple diffusion techniques (2). CED of therapeutic agents bypasses the blood–brain barrier, delivers a high concentration of therapeutic agents to the injection site, provides wider distribution of therapeutic agents within the target site, and minimizes systemic exposure, resulting in fewer side effects. However, because CED distributes therapeutic agents not only to the tumor mass but also beyond the tumor margin into normal surrounding brain tissue, the need for selective tumor cytotoxic activity remains. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called Apo2L) is a promising candidate for CED infusion because it is a potent endogenous activator of the cell death pathway and preferentially kills neoplastically transformed cells. Because glioma cells have greater expression of the transcripts (DR4 and DR5) encoding the TRAIL death receptor than normal astrocytes (3), they may represent a potential target for selective killing by TRAIL. Cellular resistance to TRAIL, however, has also been reported (4). To improve the efficacy of TRAIL administration and overcome TRAIL resistance, potentially synergistic TRAIL-based combination chemotherapeutic regimens are being studied extensively in many cancers in vitro and in vivo. A recent in vitro study using U87MG human glioma cells showed the synergistic effect of TRAIL and temozolomide (TMZ; ref. 5) against tumor cells. Because TMZ is generally well tolerated, can easily be administered orally, and has been shown to cause objective response or stabilization of disease in 50% to 60% of patients with glioblastoma (6), we were encouraged to further explore the TRAIL-TMZ drug combination in vivo. In this study, we evaluated the combination therapy of CED infusion of TRAIL and systemic administration of TMZ for efficacy against a U87MG intracranial xenograft model.

Materials and Methods

Recombinant TRAIL and Temozolomide. Two recombinant human TRAIL proteins were used in this study. One was soluble recombinant human TRAIL composed of residues 114 to 281 (Calbiochem, San Diego, CA), and the other was the NH2-terminal His6-tagged recombinant human TRAIL composed of residues 95 to 281 (R&D Systems, Minneapolis, MN). All studies were done with the recombinant protein purchased from Calbiochem, except for the distribution study, which used the protein purchased from R&D Systems; to detect only the distribution of injected TRAIL protein without detecting TRAIL protein endogenously expressed by gliomas (7), His-tagged protein and anti-His tag antibody were used for evaluation of distribution. TMZ was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD) and was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO).

Tumor Cell Line. An established human glioblastoma multiforme cell line, U87MG, was obtained from the Brain Tumor Research Center Tissue Bank at the University of California, San Francisco. Cells were maintained as monolayers in a complete medium consisting of Eagle’s minimal essential medium supplemented with 10% fetal calf serum and nonessential amino acids. Cells were cultured at 37°C in a humidified atmosphere consisting of 95% air and 5% CO2.

In vitro Exposure to TRAIL and Temozolomide. On the day before treatment, 5 × 104 cells per well were seeded into a 24-well plate (Corning Inc., Corning, NY). After 24 hours of incubation, cells were exposed to TMZ (0–50 μmol/L), TRAIL (0–350 ng/mL), or both agents in serum-free medium. DMSO, which was used to dissolve TMZ, had no effect on cell growth at the concentration used (<0.1% (v/v)) in this study. After another 24 hours of incubation, cell survival was estimated using a trypan blue exclusion assay.

Western Blotting for Cleaved Caspase-8, Cleaved Caspase-3, and Actin. On the day before treatment, 80 × 103 cells per well were seeded into a 6-well plate (Corning Inc.). After 24 hours of incubation, cells were exposed to TMZ (0–50 μmol/L), TRAIL (200 ng/mL), or both agents in serum-free medium. After 6 hours of incubation, cells were collected, and protein was extracted using cell lysis buffer (Cell Signaling Technology, Beverly, MA). Equal amounts of protein were separated by 15% SDS-PAGE and blotted onto PDGF membrane (Bio-Rad, Hercules, CA). The PDGF membrane was incubated in blocking

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buffer (Tris-buffered saline/casein blocker; Bio-Rad) and then with anti-cleaved caspase-8 antibody (Cell Signaling Technology; 1:500, 4°C, overnight), anti-cleaved caspase-3 antibody (Cell Signaling Technology; 1:500, 4°C, overnight), and anti-β-actin antibody (Sigma; 1:1,000, room temperature, 1 hour). The blot was visualized with a colormetric detection kit (Opti-4CN Detection Kit; Bio-Rad).

Animals. Congenitally athymic, male, nude rats (nu/nu, homozygous) were purchased from the National Cancer Institute and housed under aspecific conditions, which included filtered air and sterilized food, water, bedding, and cages. Animals weighed approximately 250 g at the time of the experiments. All protocols used in the animal studies were approved by the University of California, San Francisco Committee on Animal Research.

Intracranial Tumor Implantation. U87MG cells were harvested by trypsinization, washed once with Hank’s balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS), and resuspended in HBSS for implantation. A cell suspension containing 5 × 10³ cells per 10 μL of HBSS was used for implantation into the striatum of rat brains. Under deep isoflurane anesthesia, rats were placed in a small animal stereotactic frame (David Kopf Instruments, Tujunga, CA). A sagittal incision was made through the skin to expose the cranium, and a burr hole was made in the skull at 0.5 mm anterior and 3 mm lateral from bregma using a small dental drill. Five microliters of cell suspension were injected at a depth of 4.5 mm from the brain surface. After a wait of 2 minutes, another 5 μL were injected at a depth of 4 mm. After a final wait of 2 minutes, the needle was removed, and the wound was closed with sutures.

Distribution of TRAIL after Convection-Enhanced Delivery. Two intact rats and two tumor-bearing rats were used to evaluate the distribution of recombinant TRAIL (His-tagged) after CED infusion into their striatum or brain tumor, respectively. Nine days after tumor implantation, TRAIL protein (2 μg per 20 μL of PBS) was infused locally by means of CED. Using the same stereotactic coordinates as used for tumor cell implantation, an infusion cannula was inserted into the brain. The infusion was performed using our CED method as described previously (8). The following ascending infusion rates were applied to achieve the 20-μL infusion: 0.2 μL per minute for 15 minutes; 0.5 μL per minute for 10 minutes, and 0.8 μL per minute for 15 minutes. Animals were euthanized 4 hours after infusion, and their brains were harvested, fixed with 10% formalin, and cut into 40-μm serial coronal sections using a cryostat. Every twentieth section was stained for immunohistochemical analysis. Sections were washed in PBS and incubated in 3% hydrogen peroxide for 20 minutes. Sections were then washed in PBS and then incubated in blocking solution (10% normal horse serum and 0.1% Triton X-100 in PBS) for 30 minutes, followed by incubation in anti-His tag antibody solution (rabbit polyclonal antibody; 1:1,000; Cell Signaling Technology) for 24 hours. The sections were then incubated for 1 hour in biotinylated antirabbit IgG secondary antibody (1:1,200; Vector Laboratories, Burlingame, CA). Antibody binding was visualized with streptavidin-horseradish peroxidase (1:1,000; Vector Laboratories) and 3,3'-diaminobenzidine chromogen (Vector Laboratories).

Evaluation of Toxicity. Three intact rats were evaluated for potential local toxicity after CED infusion of TRAIL protein. CED was performed as described above. Seven days after CED infusion of 2 μg of TRAIL protein in 20 μL of PBS into the striatum, rats were euthanized, and their brains were fixed, subjected to paraffin sectioning (5 μm), and stained with hematoxylin and eosin (H&E). Additionally, two intact rats were evaluated for potential hepatotoxicity after CED infusion of TRAIL protein. Rats received two CED infusions of 2 μg of TRAIL protein in 20 μL of PBS into the striatum (the second infusion was performed 5 days after the first CED). Seven days after the second CED, rats were euthanized, and their livers were fixed, subjected to paraffin sectioning (5 μm), and stained with H&E.

Combined Therapy against the U87MG Intracranial Xenograft Model. Thirty-five nude rats that received U87MG tumor cell implants were randomly divided into four groups: the control group (n = 8), the TMZ group (n = 9), the TRAIL group (n = 9), and the combination group (n = 9). Seven and 10 days after tumor implantation, CED infusions of 2 μg of TRAIL per 20 μL of PBS were performed in the TRAIL and combination groups (9). In the control and TMZ groups, CED infusion of 20 μL of PBS was performed as a control. In the TMZ and combination groups, TMZ (350 mg/m²/d) in a solution of 10% DMSO in 0.9% saline at a volume of 90 mL/m² was given systemically (intraperitoneal administration) daily for 5 days, starting on day 7 after tumor implantation (10). Two rats from each group were euthanized 14 days after tumor implantation, and their brains were processed in paraaffin block for detection of apoptosis. All other rats (n = 6 for the control group and n = 7 for the TMZ, TRAIL, and combination groups) were monitored for survival. Survival between the treatment groups was compared using a log-rank test. Estimated survival was expressed as a Kaplan-Meier curve.

TUNEL Staining. Paraffin sections made from the brains of the two rats from each treatment group euthanized 14 days after tumor implantation were assessed for apoptosis. DNA fragmentation [terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining] was determined by using the FD NeuroApp Kit (FD Neurotechnologies Inc., Ellicott City, MD) according to the manufacturer’s instructions. Counterstaining was performed using FD Methyl Green Solution (FD Neurotechnologies).

Results

Synergistic Cytotoxic Effects of TRAIL and Temozolomide In vitro. Synergism, as determined by isobologram analysis, was observed (11) between the two agents (Fig. 1A and B). The IC₅₀ calculated from this experiment was 314.9 ng/mL for TRAIL and 46.2 μmol/L for TMZ. Western blotting for cleaved caspase-8 and caspase-3 detected the activation of both caspases in cells treated with TRAIL (200 ng/mL) or with the combination of TRAIL and TMZ (15 μmol/L; Fig. 1C). A similar level of activation of caspase-8 was observed in cells treated with TRAIL alone and in cells treated with TRAIL and TMZ. Activation of caspase-3, however, was enhanced in cells treated with TRAIL and TMZ.

Effective Distribution of TRAIL Protein after Convection-Enhanced Delivery Infusion. Sequential sections of the rat brain following CED or TRAIL (40-μm thickness and 800-μm intervals) are shown in Fig. 2A. Robust staining for His-tagged TRAIL protein was observed, demonstrating wide distribution of protein infused by means of CED, which covered the whole striatum and surrounding white matter tissue. Seven days after infusion, moderate inflammation was found only immediately adjacent to the needle tract (Fig. 2B) in the rats used for local toxicity evaluation. No rats developed any discernable symptoms, including neurologic deficits. Rats used for hepatotoxicity evaluation also developed no clinical symptoms. Histologic examination of their livers, which were obtained 7 days after the second CED infusion (the first CED infusion was performed 5 days before the second CED infusion), found no obvious liver damage (Fig. 2C). For the rats with implanted U87MG tumors treated with CED of TRAIL 9 days after tumor implantation, positive staining for His-tagged TRAIL covered almost the entire tumor and portions of surrounding normal tissue, suggesting complete coverage of the tumor and tumor margins by TRAIL (Fig. 2D).

Combined Effect of TRAIL and Temozolomide in U87MG Brain Tumor Xenografts In vivo. All rats from the control group, which received CED infusion of PBS and intraperitoneal injection of 10% DMSO solution (the vehicle for TMZ), developed large tumors and had to be euthanized 19 to 23 days after tumor implantation. In contrast, the TRAIL group (CED infusion of TRAIL and intraperitoneal injection of 10% DMSO solution) and the TMZ group (CED infusion of PBS and intraperitoneal injection of TMZ) survived significantly longer (P < 0.01, log-rank test). Furthermore, 70% (five of seven) of the animals from the group receiving combination treatment (both TRAIL and TMZ) that were analyzed for survival lived significantly longer than the animals in the single-therapy groups (until at least 80 days, when the experiment was terminated; P = 0.006 compared with TRAIL group, P = 0.032 compared with TMZ group, log-rank test).

Histologic Findings and Detection of Apoptotic Cell Death after Treatment. Histologic examination revealed the presence of a large tumor in every rat that had to be euthanized due to neurologic symptoms after treatment (Fig. 4A, i). Seven rats survived more than 80 days: one rat each from the TRAIL and TMZ groups, and five rats

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from the combination therapy group. Careful examination of the brains of rats that received combination therapy and survived more than 80 days revealed fibrous scar tissue at necropsy (Fig. 4A, ii and iii). TUNEL staining performed on brain slices of rats from each group 14 days after tumor implantation (i.e., 3 days after treatment completion) showed that tumors from rats treated with both TRAIL and TMZ exhibited a decrease in tumor cell density (Fig. 4B, top panels) and an increase in the number of TUNEL-positive cells as compared with those from animals treated with either agent alone or control animals (Fig. 4B, bottom panel).

**Discussion**

Promising strategies for effective treatment of brain tumors have always been hampered by difficulty in drug delivery. The blood–brain barrier usually compromises the effective delivery of systemically administered drugs, and local injections that bypass the blood–brain barrier often yield poor drug distribution within the injected site. In an effort to improve drug delivery within the brain, several recent studies have demonstrated the possibilities of CED in this regard (8). Several chemotherapeutic agents delivered locally using CED, including 1,3-
bis(2-chloroethyl)-1-nitrosourea (12), gemcitabine, and carboplatin (13), provided favorable therapeutic outcomes. Although these promising strategies may warrant clinical trials, there are concerns about using highly cytotoxic agents with extensive distribution in the central nervous system. It is known that some of these drugs, when applied locally into the cerebrospinal fluid or systemically at high dose, cause long-term side effects including leukoencephalopathy and brain atrophy (14). Ideally, agents for CED administration into brain tumors would be the agents that show the highest possible therapeutic ratio against tumor cells over normal cells. Because of the selective toxicity of TRAIL against neoplastically transformed cells, it represents an excellent candidate for local delivery (15, 16). In this study, we succeeded in delivering the TRAIL protein using our CED method into the entire tumor mass and into surrounding brain areas that usually contain invading cells in patients with malignant glioma. Also, in accordance with results reported elsewhere, TRAIL toxicity against normal brain was minimal, even with enhanced distribution (9). Hepatotoxicity in response to systemic administration of TRAIL has been shown (17, 18). However, after CED of TRAIL, the majority of the drug is retained by the brain, and no obvious liver damage was observed after the local administration method used in this study. These results suggest that in TRAIL-sensitive gliomas, CED-delivered TRAIL may be a viable therapeutic option.

In addition to antitumor activity, one more promising property of TRAIL is that synergistic effects with chemotherapy may be achievable in malignant glioma cells (19). Synergism between systemically administered cis-diamminedichloroplatinum(II) and TRAIL has already been demonstrated in an experimental glioma model (19). We hypothesized that combining TRAIL-sensitizing chemotherapy with CED administration of TRAIL might improve the therapeutic index of a TRAIL-based anticancer strategy. We chose TMZ as a TRAIL sensitizer because use of TMZ is already clinically established for glioma therapy and because synergism between TRAIL and TMZ has been suggested recently in vitro (5). In this study, TMZ given systemically was shown to sensitize intracranial tumors to TRAIL-induced cell death. Thus, systemic delivery of chemotherapeutic drugs such as TMZ combined with CED administration of TRAIL may be a promising method for the treatment of malignant brain tumors. Recently, synergistic effects of recombinant TRAIL administered with chemotherapeutic agents were also demonstrated in several

Fig. 3. TRAIL, and TMZ chemotherapy, in combination, prolongs the survival of U87MG brain tumor xenografts. A, experimental design of the survival study. Daily intraperitoneal injections of TMZ (350 mg/m²/d) were performed 7 to 11 days after tumor implantation, and CED infusions of TRAIL (2 μg in 20 μL of PBS) were performed on days 7 and 10. B, Survival of treated animals was observed for 80 days and is expressed as a Kaplan-Meier curve. Six or seven rats for each group were used in the study.

Fig. 4. Histologic findings at necropsy and TUNEL staining. A, H&E staining of the brains of rats that failed to survive the observation period revealed large tumor formation (A, i), whereas in the brains of rats that received combination therapy and survived the observation period, small fibrous scar tissues were detected (A, ii and iii). B, Assessment for apoptosis was performed 3 days after completion of treatment. Tumors were sectioned and subsequently stained with H&E (top panels) or TUNEL (bottom panel). TUNEL-positive cells stain brown. Methyl green was used for counterstaining.
TRAIL-resistant cancer models. Some of the chemotherapeutic agents worked to change the death receptor profile in tumor cells, i.e., to increase the expression of death receptors DR4 and DR5 (20). Some compounds that affect the caspase pathway also worked synergistically with TRAIL (21). Radiation therapy, which has also been established as an accepted treatment modality for gliomas, may also serve as a TRAIL sensitizer for CED-delivered TRAIL (22). These findings that TRAIL sensitizers work not only in TRAIL-sensitive cells but also in TRAIL-resistant cells strongly support the importance of developing TRAIL-based combination strategies. To apply recent combination strategies using TRAIL and TRAIL sensitizers to clinical applications for brain tumor treatment, distribution of TRAIL protein throughout the tumor mass will be the most basic requirement, which is difficult to achieve in brain tumors. We believe that our CED method will help not only in the development of TRAIL-based therapy but also in other combination treatment strategies in the future.

In this study, CED of TRAIL to tumor and surrounding tumor-infiltrated brain, alone or in combination with TMZ, improved therapeutic outcome in a human glioblastoma xenograft model. We propose that the CED approach used in this study offers a new and potentially effective method to deliver novel TRAIL-based chemotherapeutic regimens.

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References

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