Induction of Glioblastoma Apoptosis Using Neural Stem Cell-mediated Delivery of Tumor Necrosis Factor-related Apoptosis-inducing Ligand

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Introduction

Despite refinements in current therapeutic strategies, the prognosis for patients with glioblastoma multiforme remains dismal (1). The futility of present treatments in combating this disease is, in part, due to their inability to address the highly invasive nature of these neoplasms. Glioblastoma cells are able to disseminate throughout the brain and intersept themselves with normal brain parenchyma, often at great distance from the primary tumor mass. This leads to the development of tumor satellites that escape resection and treatment, eventually serving as reservoirs for tumor recurrence. Targeting these tumor satellites may prove critical for the success of any potential therapeutic strategy.

NSCs have been shown to exhibit potent tropism for disseminating glioma cells in vivo (2, 3). This endows them with the capacity to specifically target microscopic tumor outgrowths and microsatellites. We have previously demonstrated that NSCs engineered to secrete interleukin 12 can track migrating tumor cells and induce cytotoxic immune responses against tumor pockets (3). To further investigate the therapeutic potential of NSCs, we tested the treatment efficacy of tumor-tracking NSCs in a nonimmune therapeutic approach involving delivery of the proapoptotic protein TRAIL to human intracranial glioma xenografts. We demonstrate that TRAIL-secreting NSCs can migrate to tumor satellites distant from the primary tumor mass and induce potent apoptotic activity in both the main tumor and tumor pockets, leading to a highly significant reduction in tumor volume. These results further support the viability of tumor-tracking NSCs as a platform for therapeutic protein delivery to intracranial brain tumors.

Abstract

Current therapies for gliomas fail to address their highly infiltrative nature. Standard treatments often leave behind microscopic neoplastic reservoirs, resulting in eventual tumor recurrence. Neural stem cells (NSCs) are capable of tracking disseminating glioma cells. To exploit this tropism to develop a therapeutic strategy that targeted tumor satellites, we inoculated human glioblastoma xenografts with tumor necrosis factor-related apoptosis-inducing ligand-secreting NSCs. This resulted in the dramatic induction of apoptosis in treated tumors and tumor satellites and was associated with significant inhibition of tumor growth. These results add credence to the potential of NSCs as therapeutically effective delivery vehicles for the treatment of intracranial glioma.

Materials and Methods

Cell Culture and Virus. The U343MG cell line (human glioblastoma) was the kind gift of Dr. Chunhai Hao (Department of Pathology, University of Alberta, Edmonton, Alberta, Canada) and was grown in DMEM/Ham’s F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 2 mM L-glutamine, 100 µg/ml penicillin, and 100 units/ml streptomycin (Invitrogen). NSCs, harvested from the frontal-parietal regions of day 15 fetal C57Bl/6 mice as described previously (4), were cultured in DMEM/Ham’s F-12 supplemented with B-27 supplement (Invitrogen), 20 ng/ml murine epidermal growth factor, and 20 ng/ml human basic fibroblast growth factor (Peprotech, Rocky Hill, NJ). The recombinant replication-deficient adenoviral vectors bearing the genes for β-galactosidase (AdLacZ) and human TRAIL (AdTRAIL) were constructed as described previously (5, 6).

In Vitro Infection of NSCs and U343MG Cells with Adenoviral Vectors. NSCs and U343MG cells were infected with 100 MOI of either AdTRAIL or AdLacZ. Twenty-four h after infection, supernatant was collected and tested for TRAIL content using an ELISA (BD Pharmingen, San Diego, CA), and cells were stained for β-galactosidase using a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside substrate as per standard protocol. Before in vivo inoculation, all virally infected NSCs were washed three times with PBS (pH 7.4; ~50 ml/wash) to ensure that final inoculates were devoid of free viral particles.

Detection of in Vivo Apoptosis and Assessment of Cell Viability. U343MG cells and NSCs were stained 24 h after either adenoviral infection or exposure to rTRAIL (30 ng/ml; Peprotech) with a TUNEL assay (Roche, Mannheim, Germany). Quantitation of cell viability was performed using a colorimetric assay for mitochondrial dehydrogenase activity (WST-1; Roche) as described previously (7).

Inoculation of Established Intracranial Gliomas with NSCs. Athymic nude mice (nu/nu; 6–8 weeks old; Charles River Laboratories, Wilmington, MA) were anesthetized with i.p. ketamine and xylazine and stereotactically inoculated with 10⁵ U343MG cells in 5 µl of 1.2% methylcellulose/MEM in the right corpus striatum. At day 7 postimplantation, the animals were divided into three groups and treated with intratumoral inoculations of saline (5 µl; n = 5), 2 × 10⁵ TRAIL-secreting NSCs (NSC-TRAIL; n = 6), or 2 × 10⁵ β-galactosidase-expressing NSCs (NSC-LacZ; n = 6), in 5 µl of serum- and virus-free medium, injected directly into the established tumor using the same burr hole and stereotactic coordinates. All animal use was performed in strict accordance with Animal Care and Use Committee guidelines in force at Cedars-Sinai Medical Center.

Immunohistochemistry and Detection of in Vivo Apoptosis. Brains harvested from treated mice were frozen on dry ice, sectioned using a cryostat, mounted on slides, and then fixed in acetone. Staining was performed using primary antibodies against β-galactosidase (1:100; MAB1802; Chemicon, Temecula, CA) and TRAIL (1:25; MAB375; R&D Systems, Minneapolis, MN). Secondary staining was then performed with the Vectastain M.O.M. Immunodetection kit (Vector Laboratories, Burlingame, CA). Slides were developed with diaminobenzidine (Sigma, St. Louis, MO) and counterstained with hematoxylin before final mounting. To detect in vivo apoptotic activity, tumor-bearing brain sections from treated animals were mounted on slides and fixed with 4% paraformaldehyde. Staining was then performed using a TUNEL assay kit (Roche) and developed using the Vector Red Substrate Kit (Vector Laboratories) or BCIP/NBT (Sigma). Vector Red-developed TUNEL-
stained slides were lightly counterstained with hematoxylin before final mounting. BCIP/NBT-developed slides were not counterstained.

**Determination of in Vivo Tumor Size.** One week after therapeutic intratumoral inoculations, mice were euthanized using CO₂ asphyxiation, and their brains were harvested and frozen immediately on dry ice. Brains were sectioned using a cryostat into 10-μm-thick slices that were mounted on slides and then stained with H&E as per standard protocol. Tumor size was determined as described previously (8, 9), by making serial H&E-stained coronal sections spaced 150 μm apart from three to four brains belonging to each treatment group. The section bearing tumor with the maximum visible diameter was identified and scanned into a computer. The NIH Image software package was then used to calculate tumor area on the scanned section. Visible tumor boundaries were demarcated on the screen, and the software package calculated the number of pixels in the delineated area. The pixel count was then normalized to the original dimensions of the scanned sections, and the tumor area (in mm²) was derived.

**Results**

**TRAIL Induces Apoptosis in Glioma Cells but not in NSCs.** To determine the effect of TRAIL on U343MG cells and NSCs, we cultured these cells for 24 h in media containing 30 ng/ml rTRAIL. As illustrated in Fig. 1, U343MG cells rapidly underwent apoptosis, and cultures were almost completely dead within 24 h. In contrast, NSCs

![Fig. 1. TRAIL induces apoptosis in U343MG cells but not in NSCs. U343MG cells grown with rTRAIL, AdTRAIL, or in coculture with NSC-TRAIL exhibited significant cell death 24 h after exposure, as visible under bright-field phase-contrast microscopy (left column). These cultures also demonstrated numerous TUNEL-positive nuclei, indicating that the majority of cells were undergoing apoptosis (center column). In contrast, NSCs exposed to rTRAIL or infected with AdTRAIL demonstrated negligible apoptotic activity. Similarly, U343MG cells infected with the control vector AdLacZ or grown in coculture with NSC-LacZ also remained viable. Right column represents a 4',6-diamidino-2-phenylindole (DAPI) counterstain for nuclei. In each row, the TUNEL and 4',6-diamidino-2-phenylindole images represent identical fields. Bright-field images were obtained from separate visual fields. Magnification: left column, ×100; center and right columns, ×200.](image-url)
In 30 ng/ml TRAIL-containing media remained viable and did not undergo apoptosis. To evaluate the effect of TRAIL gene transfer, we infected both U343MG cells and NSCs with 100 MOI of AdTRAIL. Twenty-four h later, U343MG cultures were largely apoptotic, whereas NSC cultures remained viable. We then wished to determine whether TRAIL secretion from NSCs could induce apoptosis in U343MG cells. NSC/TRAIL and U343MG cells were cocultured for 24 h. This resulted in significant apoptosis in U343MG cells (Fig. 1), confirming that NSC/TRAIL secreted biologically relevant quantities of TRAIL protein. U343MG cells infected with AdLacZ or cocultured with NSC-LacZ or mock-infected NSCs did not demonstrate any significant apoptotic activity. To quantitatively assess the effect of TRAIL protein and gene transfer on U343MG cells and NSCs, we performed a WST-1 mitochondrial dehydrogenase-based viability assay on cells that had been cultured with rTRAIL or infected with AdTRAIL or AdLacZ. There was a significant decrease in U343MG viability in cultures that were supplemented with rTRAIL or infected with AdTRAIL. U343MG cells exposed to rTRAIL and infected with AdTRAIL exhibited 38.8 ± 8.6% and 47.25 ± 5.1% viability, respectively, compared with untreated controls (Fig. 2A). In contrast, NSCs did not demonstrate any decrease in viability with either exposure to TRAIL protein or TRAIL gene transfer (data not shown and Fig. 2B, respectively). The decrease in cell viability seen in U343MG cells treated with rTRAIL or AdTRAIL was highly significant (Student’s t test; \( P = 0.00009 \), rTRAIL versus sham-treated control; \( P = 0.00006 \), AdTRAIL versus sham-treated control).

**In Vitro and in Vivo Expression of β-Galactosidase and TRAIL by Infected NSCs.** Supernatant from NSCs infected with AdTRAIL was analyzed using ELISA. NSC/TRAIL were found to secrete 32.5 ± 3.9 ng TRAIL/10^6 cells/24 h. In addition, NSCs infected with AdLacZ were stained in *vitro* with a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside substrate and found to express high levels of β-galactosidase (data not shown). To assess the effect of our viral vectors on NSC viability, we followed both NSC-LacZ and NSC/TRAIL *in vitro* and found them to maintain a viability in excess of 90% (as determined by trypan blue exclusion staining) up to 10 days after viral infection (data not shown). We then wished to determine whether *in vivo* transplanted NSCs continued to produce the specific gene products they had been engineered to express. Tumor-bearing brain sections from NSC/TRAIL- and NSC-LacZ-treated animals were stained for β-galactosidase and TRAIL. Numerous β-galactosidase-positive NSCs were visible within NSC-LacZ-treated tumors (Fig. 3, A–C). These cells could be seen extending into outgrowths from the main tumor mass (Fig. 3, B–D). NSC/TRAIL-treated brains demonstrated strong staining for TRAIL within inoculated tumors, tumor outgrowths, and tumor satellites, indicating the presence of TRAIL-secreting NSCs within the main tumor mass and in distant tumor pockets (Fig. 3, E–G). TRAIL expression in NSC-LacZ- or saline-inoculated brains was comparatively negligible (data not shown).

NSC/TRAIL Transplantation Induces Apoptosis in Established Intracranial Brain Tumors and Tumor Satellites. To evaluate the ability of NSC/TRAIL therapy to induce apoptosis in established intracranial gliomas, we performed TUNEL staining on tumor-bearing brain sections from inoculated animals. NSC/TRAIL-treated tumors were almost completely apoptotic (Fig. 3, H and I), indicating that TRAIL secretion from transplanted NSCs was inducing rapid and almost total tumor cell death *in vivo*. In addition, significant apoptotic activity was also visible in tumor pockets distant from the main tumor mass (Fig. 3, J and K). Importantly, apoptosis was confined to the main tumor mass and tumor pockets, with no involvement of normal brain parenchyma. TUNEL-labeled fragmented DNA could be visualized within apoptotic tumor cell nuclei (Fig. 3L). No apoptotic activity was detectable in NSC-LacZ (Fig. 3, M and N)- or saline (data not shown)-treated tumors.

**NSC/TRAIL Therapy Is Associated with Significantly Decreased Tumor Size.** We wished to ascertain whether the potent induction of apoptosis associated with NSC/TRAIL therapy would translate into tumor growth inhibition *in vivo*. Maximal tumor surface areas were determined from glioma-bearing brain sections harvested from mice euthanized 7 days after NSC inoculation. Differences in tumor size between treated groups are illustrated in Fig. 4. The average maximal tumor areas in NSC-LacZ- and saline-treated animals were 2.1 ± 0.4 and 1.9 ± 0.36 mm², respectively, compared with only 0.4 ± 0.29 mm² in NSC/TRAIL-inoculated brains. This decrease in tumor size associated with NSC/TRAIL treatment was highly significant (Student’s t test; \( P = 0.0049 \), NSC-LacZ versus NSC/TRAIL; \( P = 0.0046 \), saline versus NSC/TRAIL).

**Discussion**

TRAIL is a member of the tumor necrosis factor protein superfamily, and its use in the treatment of experimental cancer has received increasing attention (10–12). The attractiveness of TRAIL as a therapeutic agent lies in its ability to selectively induce apoptosis in a variety of neoplastic cells without causing toxicity to normal cells (11, 12). Resistance to TRAIL-induced cell death may be mediated by the inhibition of cytoplasmic caspase 8 cleavage by the apoptosis-inhibitory proteins cFLIP and PED/PEA-15, a mechanism that may be

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*NSC-MEDIATED TRAIL THERAPY FOR GLIOMA*

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Fig. 2. Exposure to TRAIL results in decreased viability in U343MG cells but not in NSCs. The viability of U343MG cells and NSCs was evaluated using a colorimetric assay 24 h after exposure to TRAIL. Viability was significantly decreased in U343MG cells exposed to either 100 MOI of AdTRAIL or 30 ng/ml rTRAIL (\( P = 0.00006 \) and \( P = 0.00009 \), respectively, versus mock-infected U343MG, Student’s t test). In contrast, NSCs infected with AdTRAIL exhibited no change in viability compared to mock-infected controls (\( P = 0.6 \), Student’s t test). NSCs exposed to 30 ng/ml TRAIL protein also demonstrated no decrease in cell viability (data not shown). Data from uninfected, unmanipulated parallel cultures of U343MG cells and NSCs represented 100% viability.

Fig. 3. Expression of β-galactosidase and TRAIL. Numerous β-galactosidase-positive NSCs were visible within NSC-LacZ-treated tumors (Fig. 3, A–C). These cells could be seen extending into outgrowths from the main tumor mass (Fig. 3, H and I), indicating that TRAIL secretion from transplanted NSCs was inducing rapid and almost total tumor cell death *in vivo*. In addition, significant apoptotic activity was also visible in tumor pockets distant from the main tumor mass (Fig. 3, J and K). Importantly, apoptosis was confined to the main tumor mass and tumor pockets, with no involvement of normal brain parenchyma. TUNEL-labeled fragmented DNA could be visualized within apoptotic tumor cell nuclei (Fig. 3L). No apoptotic activity was detectable in NSC-LacZ (Fig. 3, M and N)- or saline (data not shown)-treated tumors.

Fig. 4. Tumor-bearing brain sections were evaluated using ELISA. NSC-TRAIL were found to secrete 3.9 ng TRAIL/10⁶ cells/24 h. In addition, NSCs infected with AdLacZ were stained in *vitro* with a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside substrate and found to express high levels of β-galactosidase (data not shown). To assess the effect of our viral vectors on NSC viability, we followed both NSC-LacZ and NSC/TRAIL *in vitro* and found them to maintain a viability in excess of 90% (as determined by trypan blue exclusion staining) up to 10 days after viral infection (data not shown). We then wished to determine whether *in vivo* transplanted NSCs continued to produce the specific gene products they had been engineered to express. Tumor-bearing brain sections from NSC/TRAIL- and NSC-LacZ-treated animals were stained for β-galactosidase and TRAIL. Numerous β-galactosidase-positive NSCs were visible within NSC-LacZ-treated tumors (Fig. 3, A–C). These cells could be seen extending into outgrowths from the main tumor mass (Fig. 3, B–D). NSC/TRAIL-treated brains demonstrated strong staining for TRAIL within inoculated tumors, tumor outgrowths, and tumor satellites, indicating the presence of TRAIL-secreting NSCs within the main tumor mass and in distant tumor pockets (Fig. 3, E–G). TRAIL expression in NSC-LacZ- or saline-inoculated brains was comparatively negligible (data not shown).

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**Discussion**

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missing in TRAIL-sensitive neoplastic cells (13). Additionally, it has been postulated that TRAIL-resistant cells express either defective or decoy cell surface receptors that fail to trigger a downstream intracellular apoptotic cascade (14, 15).

The sensitivity of human gliomas to TRAIL has been demonstrated previously (16–19). Roth et al. (18) described prolonged survival in U87 glioma-bearing nude mice treated intracranially with rTRAIL. In addition, Lee et al. (19) have recently reported the use of a TRAIL gene-bearing adenovirus for in situ gene therapy of intracranial brain tumors, with encouraging results. These studies, although supportive of the tumoricidal potential of TRAIL against glioma, have limited clinical relevance. The inoculation of high-dose rTRAIL in patients is precluded by potential issues of toxicity and subtherapeutic protein half-life, whereas the use of intracranial adenoviral-based gene therapy is confronted by a range of safety issues including the danger of excessive antiviral host immune responses (20). In addition, these therapies, although capable of targeting the primary tumor mass, would be ineffective against tumor microsatellites, greatly limiting their potential to serve as effective treatment modalities for malignant glioma.

Fig. 3. Immunohistochemistry for the presence of NSCs, in vivo secretion of TRAIL, and detection of apoptosis in NSC-TRAIL-treated gliomas. NSC-LacZ or NSC-TRAIL were inoculated into established intracranial U343MG gliomas. After 7 days, treated brains were harvested and stained for β-galactosidase, TRAIL, and TUNEL. β-Galactosidase and TRAIL stains were developed using diaminobenzidine (brown), whereas TUNEL staining was developed using either Vector Red (red) or BCIP/NBT (blue). A, numerous β-galactosidase-positive NSC-LacZ within a treated tumor (T, demarcated by arrowheads). B, another tumor illustrating outgrowth from the primary tumor site into adjacent normal tissue. NSC-LacZ (brown) are visible tracking and following the tumor outgrowth. C, a higher magnification image of the outgrowth from the main neoplasm illustrated in B, illustrating numerous β-galactosidase-positive NSCs tracking disseminating tumor cells. D, a higher magnification of the boxed area in C. NSC-LacZ are visible within the tumor outgrowth. E, section from a NSC-TRAIL-treated brain demonstrating positive staining for TRAIL (brown) in both the primary tumor (T) and a tumor satellite (t). F, a higher magnification of box 1 from E. Numerous TRAIL-positive cells are visible migrating away from the primary tumor mass. G, a higher magnification of box 2 from E. Numerous TRAIL-positive cells are visible within the tumor satellite at considerable distance from the primary tumor mass, indicating the presence of NSC-TRAIL within the tumor pocket. Staining represents the presence of intracellular TRAIL in NSC cytoplasm as well as secreted extracellular TRAIL. H, a NSC-TRAIL-treated tumor stained with TUNEL and developed with Vector Red demonstrating almost complete cellular apoptosis in the main tumor mass (T). I, a higher magnification of the boxed area in H, demonstrating specificity of staining in the apoptotic tumor and lack of apoptosis in adjacent normal tissue. J, section from another TUNEL-stained tumor-bearing brain treated with NSC-TRAIL. Note the highly apoptotic primary tumor mass (T, demarcated by arrowheads). Significant apoptosis is also visible within a tumor satellite (t) at considerable distance from the main tumor mass. K, a higher magnification of the box in J, demonstrating specificity of TUNEL staining in the tumor satellite. L, high-power photomicrograph of TUNEL-stained apoptotic tumor cell nuclei from NSC-TRAIL-treated brain. Staining was developed with BCIP/NBT in the absence of a counterstain. Note the presence of fragmented clumps of DNA (blue) within apoptotic nuclei (Nu). M, tumor-bearing brain section from an animal treated with NSC-LacZ. Section has been stained for TUNEL and demonstrates negligible staining in the main tumor mass (T, demarcated by arrowheads). N, a higher magnification of the boxed area in M, illustrating the absence of TUNEL staining in the NSC-LacZ-inoculated tumor mass.
We (3) and others (2) have previously described the potent ability of NSCs to track disseminating glioma in vivo. After intratumoral inoculation, NSCs can be found interspersed in tumor microsatellites at considerable distance from the main tumor mass (3). Additionally, NSCs injected contralateral to an established glioma can migrate across the brain and intersperse themselves within the tumor tissue (3). These findings make NSCs particularly attractive candidates for therapeutic protein delivery because they can effectively target the primary tumor mass, tumor outgrowths, and distant tumor pockets.

In this study, we now demonstrate that the inoculation of NSC-TRAIL into established human glioma xenografts resulted in the potent induction of tumor cell apoptosis. Strong secretion of TRAIL into established human glioma xenografts resulted in the potent induction of tumor cell apoptosis. TRAIL into established human glioma xenografts resulted in the potent induction of tumor cell apoptosis. Strong secretion of TRAIL into established human glioma xenografts resulted in the potent induction of tumor cell apoptosis.

Acknowledgments

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