

Tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis in established and primary glioma cell lines

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Object. Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF cytokine family, which mediates programmed cell death (apoptosis) selectively in tumor cells. The selective tumoricidal activity of TRAIL is believed to be modulated by agonistic (DR4 and DR5) and antagonistic receptors (DcR1 and DcR2), which appear to compete for ligand binding. Because TRAIL is expressed in a wide range of tissues, including brain, kidney, and spleen, and seems consistently to induce cell death in tumor cells, the cytokine has been identified as a promising approach for selectively inducing tumor cell death. In this study, the authors examine the importance of TRAIL’s receptors in both its selectivity for tumor cells and its ability to induce apoptosis.

Methods. The authors first examined sensitivity to TRAIL and expression of TRAIL receptors in four established and four primary cultured glioma cell lines by using viability and fluorescent apoptosis assays. They then evaluated DR5 expression and JNK, caspase 3, and caspase 7 activation by conducting immunoblot analyses. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed to study expression of DR4, DR5, DcR1, and DcR2. The DR5 transcripts from one TRAIL-sensitive, one partially TRAIL-resistant, and one TRAIL-resistant cell line were subsequently sequenced. The expression of TRAIL receptors in normal and glial brain tumor pathological specimens were then compared using immunohistochemistry. Finally, to study the direct effects of DR5 on glioma cells, the authors conducted transient and stable transfections of the full-length DR5 transcript into glioma cells with and without preestablished overexpression of the antiapoptotic gene *bcl-2*.

The established glioma cell lines T98G and U87MG, and all primary cell lines, were apoptotic at greater than or equal to 100 ng/ml TRAIL. The A172 cells, by contrast, were susceptible only with cycloheximide, whereas U373MG cells were not susceptible to TRAIL. The JNK, caspase 3, and caspase 7 activity evaluated after treatment with TRAIL showed that TRAIL-sensitive cell lines exhibited downstream caspase activation, whereas TRAIL-resistant cells did not. The DR5 sequences in T98G, A172, and U373MG cell lines were identical to published sequences despite these differences in sensitivity to TRAIL. The RT-PCR performed on extracts from the eight glioma cell lines showed that all expressed DR5. Immunohistochemistry revealed ubiquitous expression of DR5 in glioma specimens, with an associated lack of decoy receptor expression. Normal brain specimens, by contrast, stained positive for both DR5 and DcR1. Overexpression of DR5 under both transfection conditions resulted in cell death in all three cell lines. The previously seen resistance of U373 cells to TRAIL was not observed. Apoptotic cell death was confirmed using DNA fragmentation in T98G cell lines and fluorescent microscopy in all cell lines. The T98G cells stably transfected with *bcl-2* before DR5 overexpression were protected from cell death.

Conclusions. The authors conclude that DR5 represents a promising new approach to directly activating the intrinsic caspase pathway in glioma cells. The fact that TRAIL-resistant gliomas do not express decoy receptors suggests a mechanism of resistance unique from that proposed for normal tissues. The overexpression of DR5 induced apoptotic cell death in glioma cells without TRAIL and was able to overcome the resistance to TRAIL demonstrated in U373 cells. The Bcl-2 protects cells from DR5 by acting downstream of the receptor, most likely at the level of caspase activation.

KEY WORDS • apoptosis • glioma • tumor necrosis factor–related apoptosis-inducing ligand

Apoptosis is the genetically regulated process through which organisms selectively eliminate unwanted cells. Unlike necrosis, once a cell commits to undergo apopto-

sis, it undergoes an organized sequence of degradation, externalization, and proteolysis, which is controlled by compartmentalized membrane-bound organelles, as well as cysteine proteases known as caspases.² The orderly nature of apoptosis is essential for normal embryonic development, tissue remodeling, tumor regression, and immune function.

Several cellular receptors have been identified as playing a major role in the activation of apoptosis.¹⁹ One of the earliest identified receptors, CD95, belongs to a cytokine family that includes nerve growth factor, TNF, and their corresponding receptors. Because human gliomas preferentially express CD95, the CD95 ligand has long been targeted as a promising means by which to induce apoptosis

Abbreviations used in this paper: cDNA = complementary DNA; FBS = fetal bovine serum; GBM = glioblastoma multiforme; HRP = horseradish peroxidase; IgG = immunoglobulin G; mRNA = messenger RNA; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium reagent; PBS = phosphate buffered saline; RIPA = radioimmunoprecipitation buffer; RT-PCR = reverse transcriptase–polymerase chain reaction; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium-polyacrylamide gel electrophoresis; TNF = tumor necrosis factor; TRAIL = TNF–related apoptosis-inducing ligand.

in tumor cells.⁷ The drawback to CD95 therapy, however, is that the cytokine has been shown to be highly toxic in animal studies.¹⁴

The search for a less toxic method of inducing apoptosis in tumor cells prompted the investigation of another TNF family ligand, TRAIL. The TRAIL is a transmembrane cytokine, with a high sequence homology to CD95.¹² Like CD95, TRAIL has been shown to induce apoptosis in tumor cell lines via caspase activation. Unlike CD95, however, TRAIL has low toxicity *in vivo* and has been shown to be well tolerated in laboratory animals.⁵

The TRAIL has been shown to induce apoptosis through the activation of intracellular death domains on the two known TRAIL receptors, DR4 and DR5.^{9,13,16,19} Two other receptors for TRAIL, DcR1 and DcR2, do not contain the intracellular death domain and do not induce apoptosis when bound to TRAIL.^{14,15} These “decoy” receptors are found in most normal tissues, apparently conferring resistance against TRAIL-induced apoptosis. Interestingly, most cancerous cells do not express decoy receptors and have been shown to be susceptible to TRAIL-induced cell death.^{13,15,20}

The two TRAIL receptors, DR4 and DR5, contain an intracytoplasmic death domain common to the TNF receptor family. The death domain of the other receptors in the TNF family, TNFR1 and Fas, interacts with residues on the adaptor proteins FADD and TRADD, which in turn activates a caspase-dependent cascade leading to cell death. Unlike TNFR1 and Fas, DR4 and DR5 have not been shown to induce apoptosis through FADD but are thought to induce apoptosis via a caspase-dependent pathway.^{9,19}

The TRAIL mRNA has been shown to be present in a several tissues, including spleen, prostate, and lung.²¹ The TRAIL is a potent inducer of apoptosis, capable of killing melanoma cells that are resistant to the effects of other TNF family cytokines. In human lymphoid cells, expression of TRAIL was found in activated CD4+ and CD8 T cells, suggesting that it participates in T cell-mediated induction of apoptosis.⁷ In contrast, several different types of nontumor-related tissue have been shown to be resistant to TRAIL-mediated apoptosis, and TRAIL is nontoxic in mice. These findings suggest that TRAIL administration could be a promising method of selectively inducing tumor cell apoptosis. The use of TRAIL is particularly intriguing in gliomas because these tumors have been shown to be resistant to many types of chemotherapy, resulting in poor patient survival.²⁰

In early studies characterizing the TRAIL/TRAIL receptor system the authors specifically evaluated the expression of DR5 and DcR1 in a variety of healthy human adult and fetal tissues as well as some tumor cell lines.^{13,20} Pan, et al.,¹³ described expression of DR5 mRNA transcripts and lack of expression of DcR1 in healthy brain. Sheridan, et al.,²⁰ demonstrated lack of expression of DR5 and DcR1 in both adult and fetal brain samples. Expression of DR4 and DcR2 in healthy brain tissue has not been directly addressed to date.

The goals in this study were: 1) to examine the susceptibility of both established glioma cell lines and primary cultures of fresh malignant glioma tissue to TRAIL; 2) to detect expression of TRAIL and the four TRAIL receptors

in these cell lines and in fixed tumor and normal brain specimens; 3) to confirm that TRAIL-induced apoptosis in gliomas follows a caspase-dependent pathway; 4) to examine the direct effects of DR5 on glioma cell lines independent of TRAIL; and 5) to evaluate the ability of bcl-2 to halt DR5-induced apoptotic cell death.

MATERIALS AND METHODS

Cell Lines

Established human malignant glioma cell lines U87MG, T98G, and U373MG, and primary human malignant glioma cell lines T114, T117, HC1230, and GH226 were cultured in Eagle modified essential media supplemented with 10% FBS at 37°C and 5% CO₂. The established human malignant glioma cell line A172 was cultured in Dulbecco modified essential media with 10% FBS at 37°C and 10% CO₂.

Cell Death Assays

All eight cell lines were seeded to a final concentration of 1×10^5 cells per well in 96-well culture plates and treated with TRAIL serially diluted to concentrations ranging from 1 ng/ml to 2 µg/ml. One group was exposed to cycloheximide 10 µg/ml in addition to TRAIL. Survival was assessed 24 hours after treatment using MTS and measuring 490 nm absorbance.

Based on susceptibility demonstrated by MTS assay (repeated three times and averaged), one TRAIL-sensitive cell line (T98G), one partially TRAIL-resistant cell line (A172), and one TRAIL-resistant cell line (U373MG) were examined for apoptosis and necrosis. Cells were seeded onto eight-well slide culture plates and treated with TRAIL (250 ng/ml) with and without cycloheximide (10 µg/ml) for 18 hours. Cell monolayers were treated with solutions of annexin V/propidium iodide, and then visualized by fluorescence microscopy.

Assay for JNK

To determine whether JNK is activated when cells are exposed to TRAIL, three cell lines (A172, T98G, and U373MG) were plated on six-well monolayer culture plates and subsequently treated with TRAIL (250 ng/ml) with and without cycloheximide (10 µg/ml) for 6 hours. Cells were lysed with RIPA buffer to obtain protein. Protein extract was incubated overnight with rabbit anti-JNK antibody and then immunoprecipitated with protein A sepharose beads. Beads were washed with RIPA buffer twice and then kinase buffer (50 mmol 4-[2-hydroxyethyl]-1-piperazineethane sulfonic acid, 50 mmol MgCl₂, 0.3 mol NaCl, 1 mmol dithiothreitol). The bead complexes were then incubated with P³² ATP and GST-c-Jun (substrate for JNK) for 30 minutes at 30°C. Samples were then subjected to SDS-PAGE on a 10 to 20% tris-glycine gel and electrophoretically transferred to a PVDF membrane. The JNK activation was detected using autoradiography.

Immunoblot Analysis

To detect expression of DR5 in glioma cell lines, the eight cell lines were cultured to confluence on 100-mm² culture plates. Cells were washed with PBS and then lysed

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with RIPA buffer to obtain cellular protein. After centrifugation at $\times 14000$ G, 30 μ g of each cell lysate supernatant was subjected to SDS-PAGE on a 10 to 20% tris-glycine gel, and then blotted to a PVDF membrane. The PVDF membrane was then probed with rabbit anti-DR5 polyclonal antibody. Protein was detected after incubation with goat anti-rabbit IgG conjugated to HRP, by chemiluminescence.

Immunoblot analysis was also performed on A172, T98G, and U373MG cells to detect caspase 3 and caspase 7 activity. After 6 or 12 hours of treatment with TRAIL (250 ng/ml) with and without cycloheximide (10 μ g/ml), cells were harvested and lysed as previously described. Again 30 μ g of each cell lysate was subjected to SDS-PAGE on a 10 to 20% tris-glycine gel and then electrophoretically transferred to a PVDF. Membranes were probed with either mouse anti-caspase 3 IgG or mouse anti-caspase 7 IgG. After incubation with goat anti-mouse IgG conjugated to HRP, chemiluminescence detection of activated caspase was performed.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was obtained from confluent monolayer culture of all eight cell lines by phenol-guanidine thiocyanate-chloroform extraction followed by treatment with ribonuclease-free deoxyribonuclease I, and then phenol-chloroform extraction. The cDNA was then synthesized by RT and oligo-dT priming from 5 μ g RNA in a total volume of 60 μ l, with the following RT conditions: 15 minutes/42°C, 5 minutes/99°C, and 5 minutes/5°C. Polymerase chain reaction for DR4, DR5, DcR1, DcR2, and β -actin was performed using primers and conditions described in detail elsewhere.¹⁷

Sequencing of DRS

To confirm that variability in sensitivity to TRAIL was not a result of mutations in the DR5 receptor in resistant cell lines, we cloned full-length and death-domain transcripts from one TRAIL-sensitive cell line (T98G), one partially TRAIL-resistant cell line (A172), and one TRAIL-resistant cell line (U373MG). Reverse transcription was performed as previously outlined in all three cell lines. Primers were designed to clone death domain of DR5 (forward primer: nucleotides 876–899, reverse primer: nucleotides 1357–1375) and full length DR5 (forward primer: nucleotides 139–157, reverse primer: nucleotides 1357–1375). Polymerase chain reaction was performed under the following conditions: 95°C for 60 seconds 56°C for 60 seconds 72°C for 90 seconds for 35 cycles then 72°C for 7 minutes. The PCR products were then cloned into a TA plasmid, selected for the appropriate insert (either full-length DR5 or death-domain DR5), and sequenced in the University of Maryland Biopolymer Laboratory.

Immunohistochemical Examination

Surgical specimens obtained in patients who underwent surgery at the University of Maryland Medical Center for glial tumors were provided by the Department of Pathology. A total of 64 tumor samples were graded according to World Health Organization classification as low-grade astrocytoma (Grade II; 14 male and seven female pa-

tients), anaplastic astrocytoma (Grade III; three male and seven female patients), glioblastoma (Grade IV; 11 male and 19 female patients), or oligodendroglioma (three male patients). Mean age at the time of death was 43 years for oligodendrogliomas, 26 years for low-grade astrocytomas, 50 years for anaplastic astrocytomas, and 48 years for GBMs. Healthy cerebral cortex samples were obtained from autopsy specimens at the University of Maryland School of Medicine, Department of Pathology. A total of 17 (6 male and 11 females) healthy brain specimens were analyzed. Mean age in patients in whom these healthy samples were obtained was 46 years. All tissue samples were fixed overnight in buffered formalin at 4°C and embedded into paraffin.

All of the polyclonal receptor antibodies showed cross-reactivity with human tissues. As a control, tissues were incubated with a rabbit IgG.

After deparaffinization, immunohistochemistry was performed using an immunohistochemistry kit. Tissues were blocked for 30 minutes each in Peroxide Block, and Power Block at room temperature. After blocking, all tissue samples were incubated overnight at 4°C with primary antibody, diluted 1:500 in PBS (pH 7.4). The sections were then washed in PBS for 5 minutes and incubated for 1 hour in biotinylated secondary antibody (anti-rabbit IgG). Finally, samples were incubated for 1 hour with a HRP-streptavidin conjugate at room temperature. Diaminobenzidine was the detection agent used, and sections were counterstained using hematoxylin before examination under light microscopy.

Cell Transfection

The transfection solution consisted of 2 mg cDNA, added to 100 μ l of serum-free medium and 6 μ l of lipofectamine. The mixture was allowed to incubate alone for 30 minutes at room temperature, before being added to the cells (200,000 cells incubated/well \times eight wells). The cells were then incubated with the DNA/Lipofectamine mixture for 5 hours at 37°C in 5% CO₂. This was followed by a 24 hour incubation in 10% FBS (37°C/5% CO₂).

Laddering Process

Transfected cells were trypsinized at different times after transfection (at 1, 2, 4, 8, 16, 24, 32, 40, and 48 hours). They were then resuspended in serum-free medium, and spun at 1000 rpm for 10 minutes. The supernatant was discarded, and the pellet was washed with PBS. The pellet was then resuspended with 500 μ l of hypotonic lysis buffer and kept on ice for 30 minutes. This mixture was spun at 4°C for 30 minutes at 14,000 rpm. The supernatant was retained and added to 500 μ l of phenol/chloroform/isoamyl alcohol (25:24:1). The solution was then centrifuged for 10 minutes at 14,000 rpm, and the aqueous layer was retained. This extraction procedure was repeated three times, and the aqueous solution was added to an isopropanol/5 M NaCl solution (10:1) and incubated at -70°C overnight. The DNA was then centrifuged at 14,000 rpm (4°C) for 30 minutes. The supernatant was retained and washed with 70% ethanol and then air dried. Finally, the DNA was dissolved in 25 μ l T-buffer/400 μ g/ml ribonuclease, incubated at 37°C for 2 hours, and then run on a 1% agarose gel.

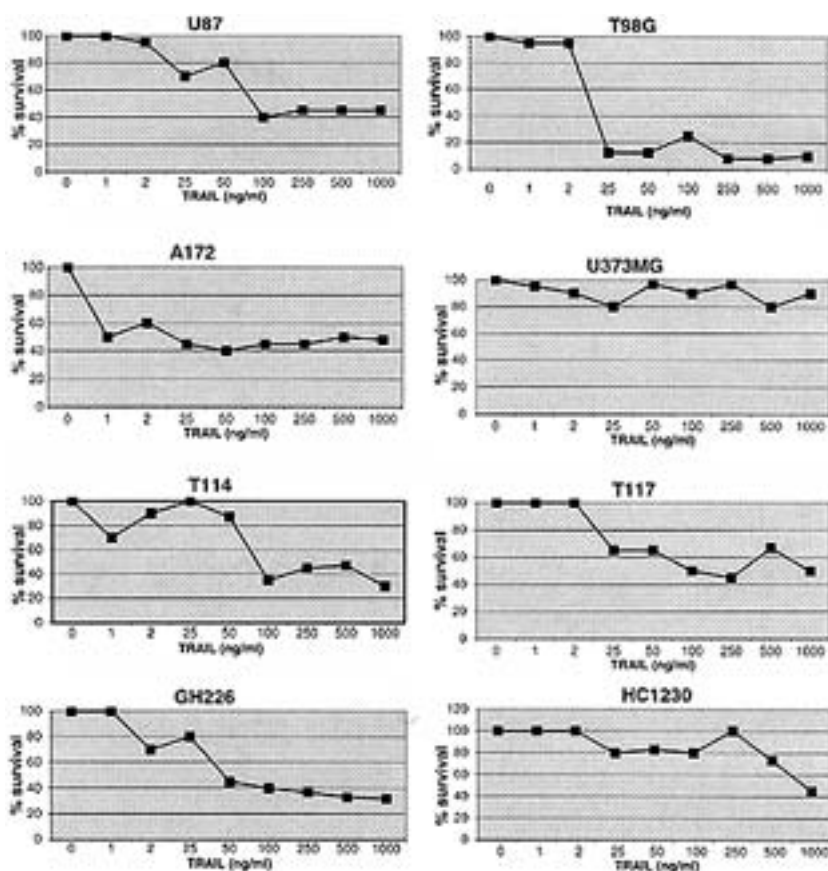


Fig. 1. Graphs showing TRAIL-induced cell death. The percentage of survival was demonstrated by MTS assay (repeated three times and averaged). Established glioma cell lines U87, T98G, A172, and U373MG were treated with escalating doses of TRAIL with or without cycloheximide. The same was conducted for the primarily cultured cell lines T114, T117, GH226, and HC1230. The assays showed that established cell lines T98G, U87MG, and A172 became apoptotic with TRAIL and cycloheximide. The U373MG cells, by contrast, were not susceptible to the effects of TRAIL even when potentiated with cycloheximide. The four primary cell lines were all susceptible to TRAIL at concentrations greater than 100 ng/ml after the addition of cycloheximide.

Sources of Supplies and Equipment

We obtained the TRAIL from Upstate Biotechnologies (Lake Placid, NY). The MTS was acquired from Promega Corporation (Madison, WI). The rabbit anti-DR5 polyclonal antibody was also manufactured by Upstate Biotechnologies. Novell Experimental Technologies (Provo, UT) is the maker of tris-glycine gel used in SDS-PAGE. We obtained the HRP from Santa Cruz Biotechnologies (Santa Cruz, CA) and the chemiluminescence device from Amersham Pharmacia Biotechnologies (Piscataway, NJ). The rabbit antibodies for DR4, Dc1, and DcR2 were obtained from Affinity Bioreagents (Golden, CO). Rabbit IgG was purchased from Vector Laboratories (Burlingame, CA). The immunohistochemistry kit (model HC-1409-06) was acquired from Innogenex (San Ramon, CA) as were the Peroxide Block, and Power Block. The detection agent, diaminobenzidine, used in immunohistochemistry was obtained from Vector Laboratories. The mouse anti-caspase 3 IgG and mouse anti-caspase 7 IgG were gifts of Dr. Sheikh (Bethesda, MD).

RESULTS

Glioma Cell Line TRAIL-Induced Apoptosis

The TRAIL sensitivity was defined as greater than 50% cell death, partial resistance as between 25 to 50% cell death, and resistance as between 0 to 25% cell death. The MTS assays showed that established cell lines, T98G and U87MG, became apoptotic at greater than or equal to 100 ng/ml TRAIL. The A172 cells became apoptotic only with the addition of cycloheximide to TRAIL. The U373MG cells were not susceptible to TRAIL-induced apoptosis. The four primary cell lines were susceptible at greater than or equal to 100 ng/ml with the addition of cycloheximide. The T114 and HC1230 cell lines were more susceptible to TRAIL alone than the other primary cell lines (Fig. 1).

Green (annexin V)-stained apoptotic cells were demonstrated in T98G cells treated with TRAIL or TRAIL combined with cycloheximide, and only in A172 cells treated with TRAIL combined with cycloheximide. The

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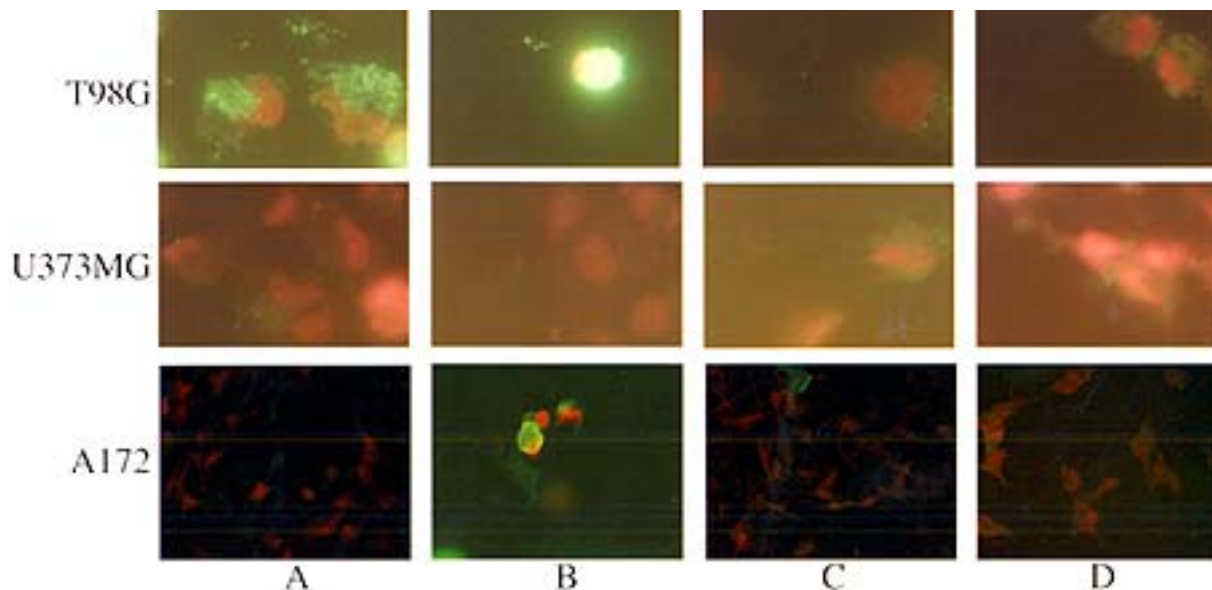


Fig. 2. Annexin V apoptosis assay. The TRAIL-sensitive (T98G), -insensitive (U373MG), and cycloheximide-enhanced TRAIL-sensitive (A172) cells were treated with TRAIL without (A) or with (B) the addition of cycloheximide, with cycloheximide alone (C), or with no agent (D) for 8 hours then exposed to Annexin V with propidium iodide counterstain. In the fluorescent photomicrographs apoptotic cells appear brilliant green, necrotic cells appear red, and viable cells appear weakly red. Green apoptotic cells are seen in T98G cells treated with TRAIL, or TRAIL combined with cycloheximide, and in A172 cells treated with TRAIL combined with cycloheximide. Consistent with the results of the MTS assay, U373MG cells showed neither apoptotic or necrotic changes. Original magnification $\times 63$.

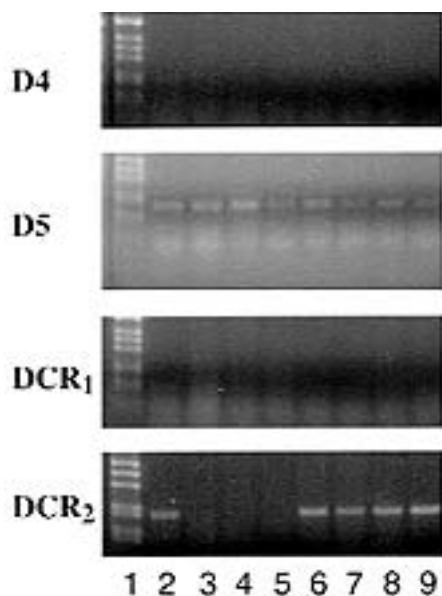


Fig. 3. A reverse transcriptase-polymerase chain reaction was performed against total RNA extracts of eight cell lines by using previously defined primers to assess the expression of the death and decoy receptors. Lane 1, standard; 2, U87; 3, T98G; 4, A172; 5, U373MG; 6, T114; 7, T117; 8, HC1230; and 9, GH226. No cell line expressed DR4 or DcR1 cDNA, whereas all cell lines expressed DR5 cDNA. Despite the fact that all four primary cell lines weakly expressed DcR2, none of the established cell lines expressed cDNA for this receptor.

U373MG cells stained neither green (apoptotic) nor red (necrotic) (Fig. 2).

The TRAIL Receptor Expression

The RT-PCR analysis was used to assess expression of DR4, DR5, DcR1, and DcR2 in both established and primary glioma cell lines. No cell line expressed DR4 or DcR1 cDNA; however, all cell lines expressed DR5 cDNA. All four primary cell lines weakly expressed DcR2, yet none of the established cell lines expressed DcR2 cDNA (Fig. 3).

Expression of DR5 was confirmed by immunoblot analysis in all eight cell lines (Fig. 4). Analysis of sequence data for full-length DR5 and DR5 death-domain transcripts from T98G (a sensitive cell line), A172 (a partially TRAIL-resistant cell line), and U373MG (a TRAIL-resistant cell line) revealed no significant mutations.

Immunohistochemical staining of pathological specimens confirmed the ubiquitous expression of DR5 in glial tumors. The distribution of immunohistochemical staining for TRAIL receptors is graphically depicted in Fig. 5, which illustrates the number of tumors with positive staining. All 64 glioma specimens, including both high- and low-grade tumors, stained positively for DR5 (Fig. 6). Three of 21 low-grade astrocytomas had DR4 staining, and no other tumor type stained for DR4. No tumors stained for either decoy receptor. In normal brain, DR5 staining was seen in all specimens, but no DR4 staining was noted. All 17 healthy brain specimens showed positive staining for DcR1 (Fig. 7), whereas three of 17 showed DcR2 staining.

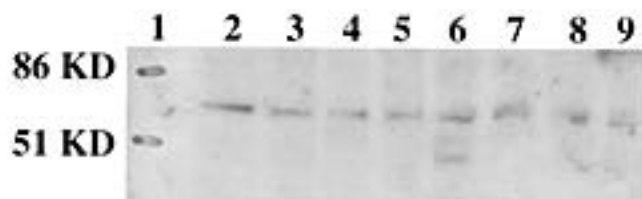


Fig. 4. The DR5 immunoblot. Cell lysate of eight glioma cell lines probed with rabbit anti-DR5 polyclonal antibody, which confirms the expression of DR5 in all eight cell lines: Lane 1, standard; 2, U87; 3, T98G; 4, A172; 5, U373MG; 6, T114; 7, T117; 8, HC1230; and 9, GH226. Despite the differing sensitivities of T98G (sensitive), A172 (partially resistant), and U373MG (resistant) to TRAIL, sequence analysis of DR5 and the DR5 death domain for these cell lines showed no significant mutations.

Caspase and JNK Activation in Response to TRAIL

The JNK activity was demonstrated in T98G cells after treatment with TRAIL or TRAIL/cycloheximide, as well as in A172 cells when treated with TRAIL combined with cycloheximide or cycloheximide alone. The JNK activity was not demonstrated in U373MG cells treated with

TRAIL, TRAIL combined with cycloheximide, or cycloheximide alone (Fig. 8).

Caspase 3 and 7 activation, detected by immunoblot analysis, was greatest in T98G cells treated with TRAIL combined with cycloheximide for 12 hours. Activation was also detected in T98G treated with TRAIL only and in A172 cells treated with TRAIL combined with cycloheximide. The U373MG cells treated with TRAIL and TRAIL plus cycloheximide did not exhibit caspase 3 and 7 activation (Fig. 9).

Transient and Stable Transfection of the Full-Length DR5 Transcript

Both transient and stable transfection of the full-length DR5 transcript were performed in T98G, A172, and U373MG cell lines. Overexpression of DR5 under both transfection conditions resulted in cell death in all three cell lines, including the TRAIL-resistant U373MG. Cell death was measured using colony counts (Fig. 10), and apoptosis was confirmed using DNA fragmentation in T98G cell lines (Fig. 11) and fluorescent microscopy in all cell lines (Fig. 12). Laddering appeared maximum at 40 to 48 hours after transfection.

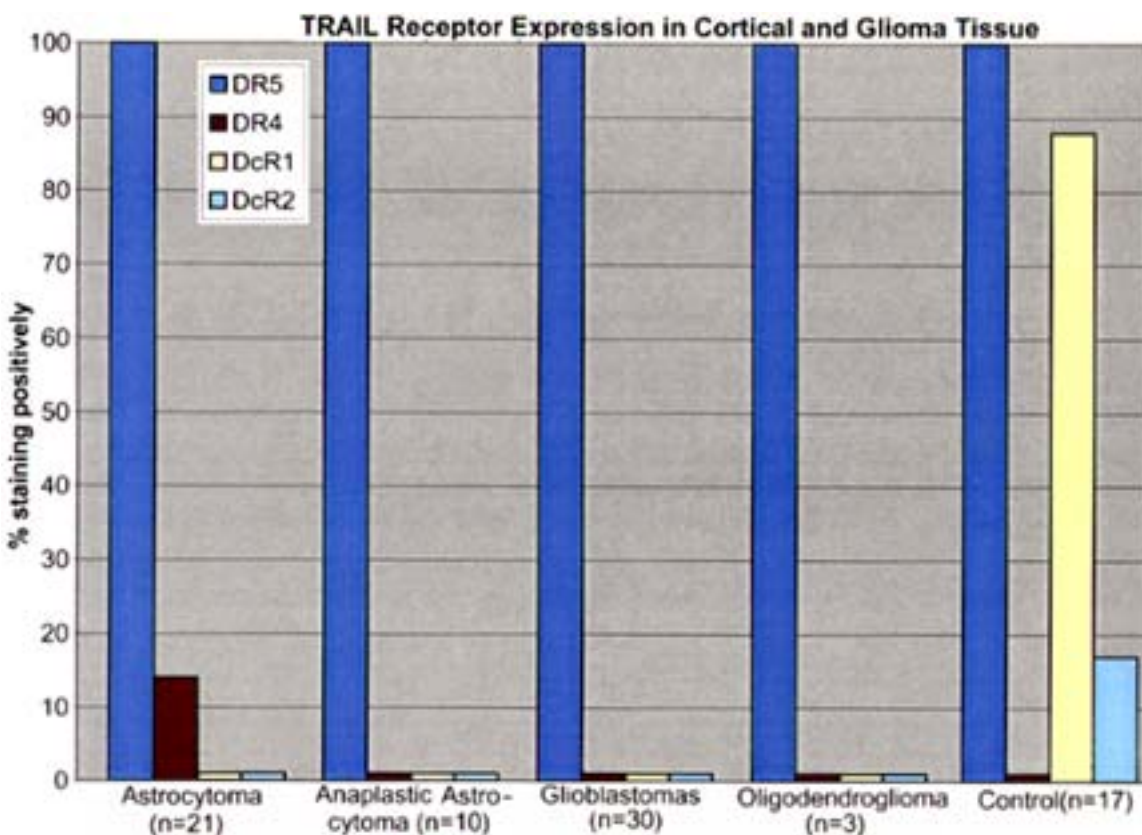


Fig. 5. Graphic showing distribution of immunohistochemical staining for TRAIL receptors. The graph depicts the percentage of positive immunohistochemical staining of TRAIL receptors in glioma and nonglioma (control/autopsy) specimens. All 64 gliomas specimens (including low- and high-grade astrocytomas) stained positively for DR5, consistent with our earlier results. Only (14%) three of 21 low-grade astrocytomas (and no other type of glioma) stained positively for DR4. No tumors demonstrated positive staining for either decoy receptor. By contrast, in healthy brain tissue, all specimens showed positive staining for DR5 as well as DcR1, whereas DcR2 only stained positively in three of 17 specimens.

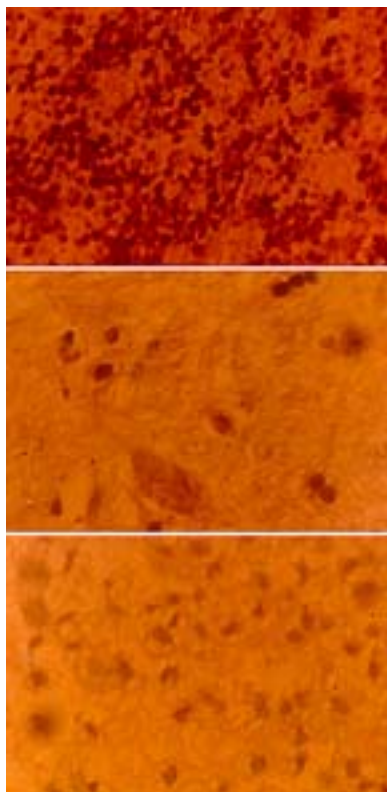


Fig. 6. Immunohistochemical staining for DR5 in malignant human gliomas. Positive staining was defined as the presence of positive staining for DR5 (using polyclonal anti-DR5 antibodies) in any section of the specimen (although most of the specimens showed diffusely positive staining). Because polyclonal antibodies were used, samples were also stained with anti-rabbit Ig as a reference for comparison. *Upper*: Human GBM stained with anti-DR5. *Center*: Noncancerous (control) autopsy specimen stained with anti-DR5. *Lower*: Human GBM stained with anti-rabbit Ig as a reference for comparison with *upper* image. As evident by comparing the *upper* and *center* photomicrographs, DR5 staining was significantly more intense in glioma specimens compared with controls, even though the control specimen did demonstrate positive staining for DR5. Original magnification $\times 310$.

The T98G Cells Stably Transfected With Bcl-2 Before DR5 Overexpression

The T98G cell lines, previously stably transfected with the antiapoptotic gene *bcl-2*, did not exhibit cell death after transfection with the full-length DR5 transcript. Furthermore, the laddering previously demonstrated in T98G cells after DR5 overexpression was no longer present (Fig. 11 *lower*).

DISCUSSION

Human malignant gliomas remain one of the most difficult tumors to treat. Because standard therapies have not significantly prolonged survival in patients, investigators have shifted focus toward manipulating tumor biology. The use of proapoptotic factors to enhance cell death has been limited by the fact that normal cells are typically susceptible to the same apoptotic factors. The TRAIL is

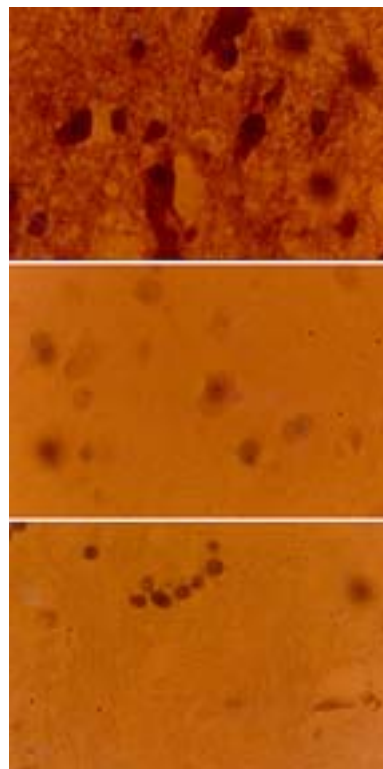


Fig. 7. Immunohistochemical staining for DcR1 in noncancerous tissue. Positive staining for DcR1 receptors (using polyclonal anti-DcR1 antibodies) was seen in a noncancerous (control) cortex (*left*), whereas the GBM (*center*) tissue specimen did not demonstrate any positive staining at all. Healthy cortical tissue (*right*) stained with anti-rabbit Ig as a reference for comparison with the photomicrograph of (*left*). This finding is consistent with the “decoy model” of TRAIL-mediated apoptosis, suggesting that cellular regulation of decoy receptors provide cells with a mechanism of modulating TRAIL sensitivity. Original magnification $\times 310$.

unique in that normal cells, perhaps via preferential expression of decoy receptors, do not appear susceptible to TRAIL. As seen in this study, human glioma cells have varying degrees of sensitivity to TRAIL. Its optimal use of TRAIL as an antitumor agent will require further understanding of its mechanism of action and the reasons for these disparities in efficacy. Results of this study will significantly add to current knowledge regarding the importance of TRAIL’s receptors in its selectivity for tumors and provides evidence that DR5 may hold the key to circumventing TRAIL’s limitations.

The TRAIL-Induced Apoptosis in Glioma Cell Lines

Similar to findings reported by Rieger, et al.,¹⁷ we found that T98G and A172 cells were sensitive to TRAIL-induced cell death and that U87 cells were sensitive only after potentiation cell death with cycloheximide. The four primary glioma explants were inhibited by TRAIL but also required cycloheximide potentiation. The U373 cells were completely resistant to TRAIL. The observation that administration of cycloheximide, an inhibitor of protein synthesis, potentiated apoptotic cell death supports the ex-

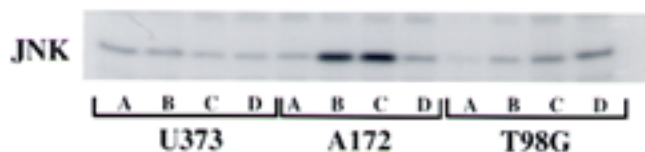


Fig. 8. The JNK activity. To demonstrate caspase and JNK activity in response to TRAIL, phosphor screen autoradiography was performed. The JNK activity was demonstrated in T98G cells when treated with TRAIL, irrespective of whether TRAIL was coadministered with cycloheximide. The A172 cells demonstrated JNK activity when treated with TRAIL and cycloheximide, or with cycloheximide alone. Significant JNK activity was not seen in U373MG cells, even when the effects of TRAIL were potentiated with cycloheximide. Therefore, JNK activity is present in TRAIL-sensitive (T98G), and cycloheximide-enhanced TRAIL-sensitive cell lines, whereas TRAIL-resistant cells show minimum activity.

istence and significance of transiently produced cellular antiapoptotic factors.

We were then able to demonstrate in glioma cells that TRAIL's activation of death receptors was correlated with JNK activity and caspase degradation, a finding consistent with recently published data, indicating that TRAIL can activate caspase 8 and downstream events of apoptosis after DR4 and DR5 activation.^{3,8} Accordingly, U373 cells, which were resistant to TRAIL, did not show JNK activation or caspase degradation. These results support a recent report in which JNK activity was linked to TRAIL-induced apoptosis in human T and B cell lines.⁶

Expression of TRAIL Receptor

To determine if the variability in response to TRAIL

was related to expression of TRAIL's receptors, we analyzed glioma cell extracts for DR4, DR5, DcR1, and DcR2 mRNA expression by using DR5 RT-PCR. Expression was detected in all eight cell lines, including the resistant glioma cell line U373. We confirmed this expression of DR5 by conducting immunoblot analysis in the same glioma cell lines, using a DR5 antibody. In our opinion, it is also significant, that DcR2 was expressed by all the primary tumor lines (as well as the U87A). This finding could be a potential roadblock in the use of TRAIL in vivo.

Using immunohistochemistry, we then examined 64 glioma and 17 normal brain specimens obtained intraoperatively for DR5 expression. In every case, we observed DR5 expression. Unlike findings in previous reports, we did not observe significant DR4, DcR1, or DcR2 expression in the glioma cell lines or surgical specimens.¹ In prior studies investigators have relied on RT-PCR techniques to determine expression, whose high sensitivities may have yielded false-positive results. All 17 healthy brain specimens stained positively for DcR1, but only three of 17 stained for DcR2. This raises the possibility that DcR1 could play a role in protecting healthy brain from TRAIL-induced apoptosis. This premise has been proposed by other groups who used PCR to demonstrate increased DcR1 expression in nontumor cell lines and in healthy human tissues such as peripheral blood leukocytes, spleen, heart, placenta, liver, lung, skeletal muscle, kidney, and pancreas.⁴

The ubiquitous expression of DR5 in glioma cell lines and tumor specimens indicates that the receptor is likely a pivotal mediator of TRAIL-induced apoptosis in glioma cells. We sequenced the DR5 receptor in both T98G (TRAIL-sensitive) and U373 (TRAIL-resistant) cell lines and found no mutations that could account for the ob-

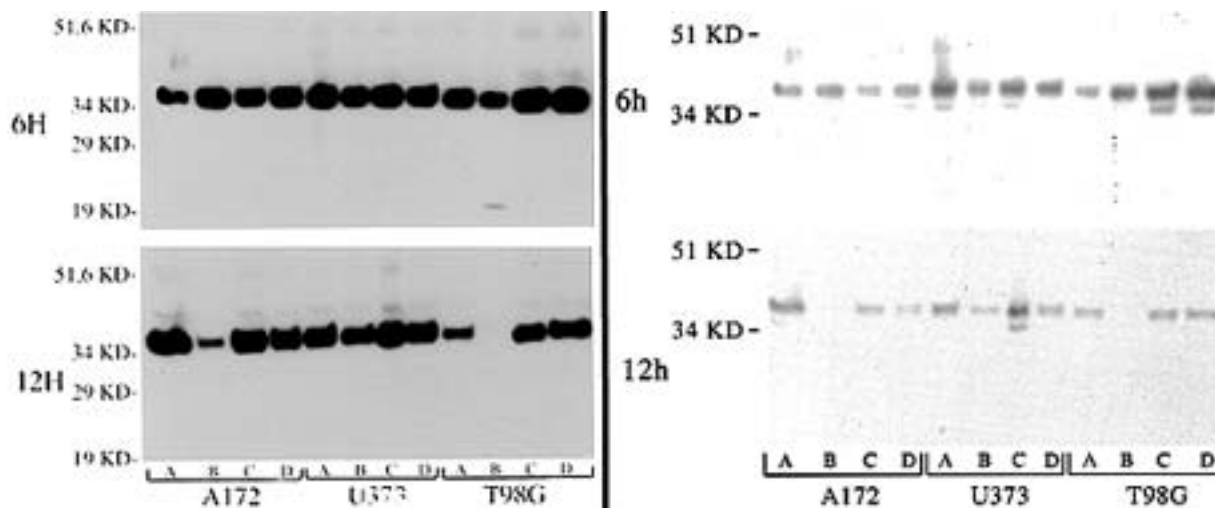


Fig. 9. Caspase 3 and 7 activity. Cell lysates of TRAIL sensitive- (T98G), -insensitive (U373MG), and cycloheximide-enhanced TRAIL sensitive (A172) cells after treatment with TRAIL alone (left) or with (right) the addition of cycloheximide, were immunoblotted with monoclonal IgG probes against the inactive form caspase 3 and 7. Activation of the caspases is shown by dropout of immunoblot signal. Caspase 3 and 7 activation was greatest in T98G cells treated with TRAIL and cycloheximide for 12 hours; activation was also seen in T98G cells treated with TRAIL alone. The U373MG cells treated with TRAIL or TRAIL/cycloheximide showed no caspase 3 and 7 activation.

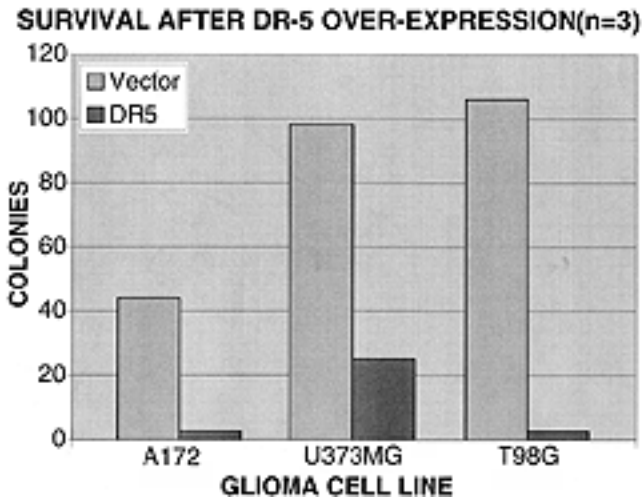


Fig. 10. Graph showing survival of cell lines after DR5 overexpression. Stable transfection of the full-length DR5 transcript was performed in T98G, U373MG, and A172 cell lines. Following overexpression of DR5, colony counts show decreased survival in T98G, A172, as well as the previously resistant U373MG cell lines. Overexpression of DR5 in transiently transfected cell lines (not depicted) showed similarly reduced survival.

served resistance. These findings indicate that the variations in sensitivity to TRAIL were not due to failed expression of DR5 transcripts or a defective DR5 receptor. Furthermore, immunoblots did not demonstrate a quantitative difference in DR5 expression. Because we found no DcR1 or DcR2 expression in our established glioma cell lines, alternate mechanisms must be demonstrated to explain the varied responses to TRAIL exposure.

The fact that U373MG cells exposed to TRAIL did not activate JNK or initiate caspase degradation suggests that the TRAIL pathway is either disrupted prior to receptor activation or between DR5 binding and initiation of the caspase cascade. The recent isolation of the decoy receptor, DcR3, which is expressed in high percentages of glioblastoma,¹⁸ and the similar decrease in clonogenic survival in both T98G and U373 cells after transfection of the full-length DR5 transcript, seem to lend credence to the former explanation.

Transient and Stable Transfection of Full-Length DR5 Transcript

The next phase of our study was to examine the direct effects of DR5 overexpression on T98G, U373MG, and U87 cell lines. As depicted in Fig. 10, all three cell lines showed reduced survival after DR5 overexpression. The therapeutic implications of this data stem from studies demonstrating that TRAIL has synergistic cytotoxic effects on tumor cells when coadministered with DNA-damaging drugs such as cisplatin or etoposide.^{10,11} These agents have been shown to induce the overexpression of DR5, which is believed to augment the cytotoxicity of TRAIL on tumor cells. Human glioma cell lines treated with cisplatin in combination with TRAIL exhibit a 100-fold greater sensitivity to TRAIL-induced cytotoxicity.¹⁰ Examination of our data, which shows that DR5 overex-

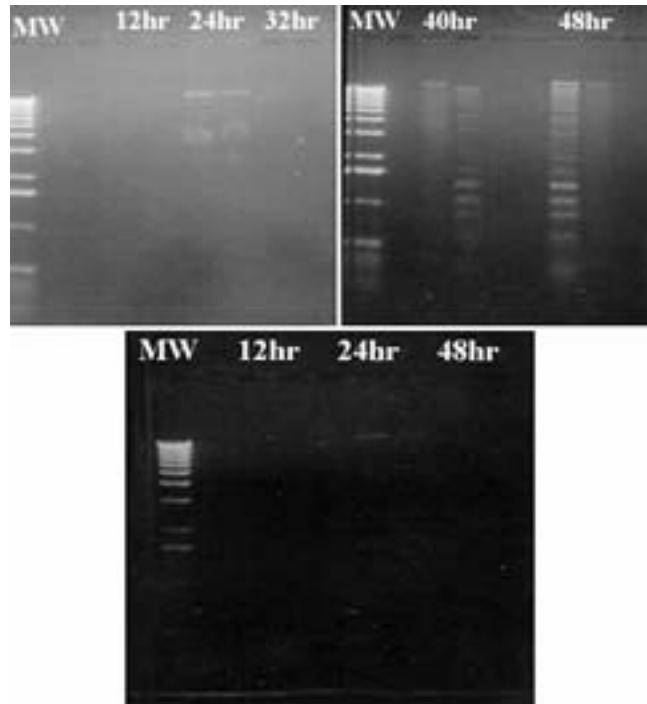


Fig. 11. Apoptosis after DR5 overexpression. In T98G cell lines, apoptosis was confirmed using DNA fragmentation. *Upper:* Time course depicting DNA fragmentation after DR5 overexpression in T98G cell lines. Laddering was best demonstrated between 40 and 48 hours after DR5 overexpression. *Lower:* No laddering, however, was seen in the same cell lines transfected with the anti-apoptotic gene bcl-2 after DR5 overexpression. These data suggest that the anti-apoptotic effect of bcl-2 occurs downstream of DR5 activation.

pression alone can activate the apoptotic pathway, suggests an alternative pathway for inducing glioma cell apoptosis without TRAIL. Overexpression of DR5 alone may avoid the T cell-mediated destruction of normal brain tissue, which has recently been identified as a possible complication of local TRAIL administration.¹²

The T98G Cells Stably Transfected With Bcl-2 Before DR5 Overexpression

The final step in our experiment was to examine the effects of DR5 overexpression in cell lines stably transfected with bcl-2. The bcl-2 family of proteins plays an important role in regulating apoptosis. This family consists of both antiapoptotic molecules, such as bcl-2 and bcl-XL, as well proapoptotic ones, such as bax and bid.¹⁸ The bcl-2 functions by blocking the release of cytochrome C from mitochondria, preventing the activation of caspase 3.

Previous data show that cell lines expressing bcl-2 are resistant to the apoptotic effects of TRAIL. In our data we found that the antiapoptotic effects of bcl-2 occurs downstream of DR5 activation, because the establishment of bcl-2 overexpression eliminated the previously observed sensitivity of T98G cell lines to DR5-mediated apoptosis. This observation was confirmed by negative immunofluorescence and DNA laddering assays. These findings con-

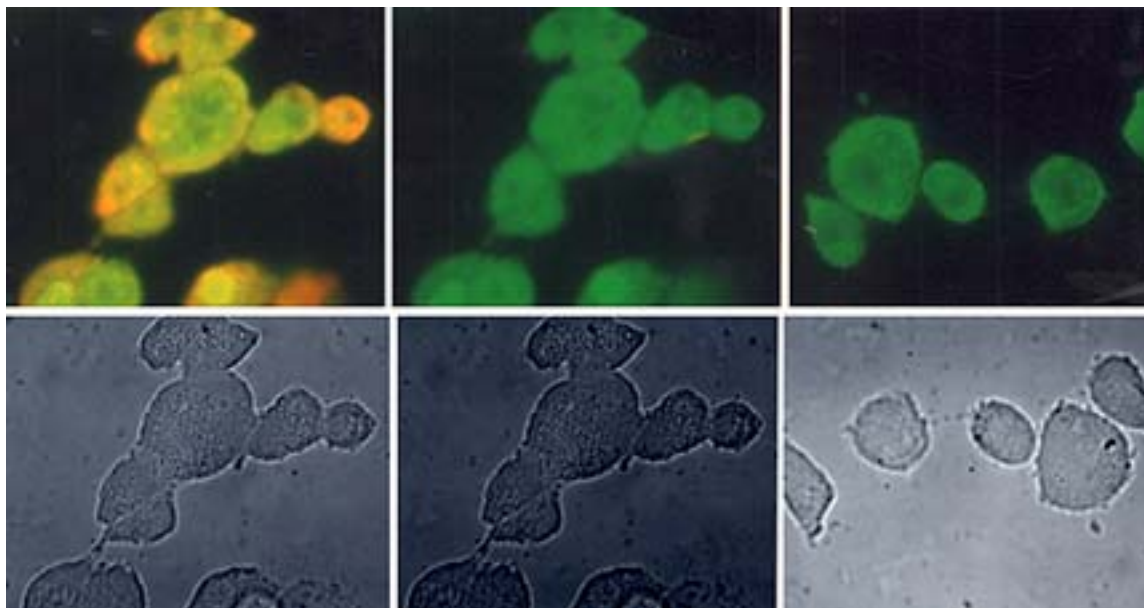


Fig. 12. The C-Jun kinase apoptosis assay after DR5 overexpression. Apoptosis demonstrated using fluorescence after DR5 overexpression in T98G (upper left), A172 (upper center), and U373MG (upper right) cell lines. Photomicrographs of the same cells, T98G (lower left), A172 (lower center), and U373MG (lower right), under light microscope are also included for reference. Apoptotic cells appeared brilliant green, while necrotic cells appeared red, and viable cells fluoresce weakly red. Although laddering was not demonstrated in the A172 and U373MG cell lines, this assay confirmed the presence of apoptotic activity in the two cell lines. Original magnification $\times 63$.

firm the inhibitory function of *bcl-2* in regulating apoptosis, suggesting that this gene is a potential target for modulating chemotherapy sensitivity in gliomas.

CONCLUSIONS

We present data that add to our understanding of TRAIL-induced apoptosis and provide further evidence to support a role for TRAIL in the treatment of human malignant gliomas. Tumor cells express the DR5 death receptor whereas normal brain tissue may be protected by the DcR1 decoy receptor. Decoy receptors were not observed in TRAIL-resistant glioma cell lines, suggesting a mechanism of resistance unique from that proposed for healthy tissues, which may occur prior to receptor activation. Cell death occurs through an apoptotic mechanism in which JNK and caspases appear to play a significant role. Overexpression of DR5 overcomes resistance in one cell line, suggesting a new potential modality for treating gliomas.

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