

Induction and Intracellular Regulation of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Mediated Apoptosis in Human Malignant Glioma Cells¹

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) preferentially triggers apoptosis in tumor cells versus normal cells, thus providing a therapeutic potential. In this study, we examined a large panel of human malignant glioma cell lines and primary cultures of normal human astrocytes for their sensitivity to TRAIL. Of 13 glioma cell lines, 3 were sensitive (80–100% death), 4 were partially resistant (30–79% death), and 6 were resistant (<30% death). Normal astrocytes were also resistant. TRAIL-induced cell death was characterized by activation of caspase-8 and -3, poly(ADP-ribose) polymerase cleavage, and DNA fragmentation. Decoy receptor (DcR1 and DcR2) expression was limited in the glioma cell lines and did not correlate with TRAIL sensitivity. Both sensitive and resistant cell lines expressed TRAIL death receptor (DR5), adapter protein Fas-associated death domain (FADD), and caspase-8; but resistant cell lines expressed 2-fold higher levels of the apoptosis inhibitor phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15 kDa (PED/PEA-15). In contrast, cellular FADD-like IL-1 β -converting enzyme-like inhibitory protein (cFLIP) expression was similar in sensitive and resistant cells. Transfection of sense PED/PEA-15 cDNA in sensitive cells resulted in cell resistance, whereas transfection of antisense in resistant cells rendered them sensitive. Inhibition of protein kinase C (PKC) activity restored TRAIL sensitivity in resistant cells, suggesting that PED/PEA-15 function might be dependent on PKC-mediated phosphorylation. In summary, TRAIL induces apoptosis in >50% of glioma cell lines, and this killing occurs through activation of the DR pathway. This caspase-8-induced apoptotic cascade is regulated by intracellular PED/PEA-15, but not by cFLIP or decoy receptors. This pathway may be exploitable for glioma and possibly for other cancer therapies.

INTRODUCTION

DRs³ of the TNF family can induce apoptosis, and activating these receptors specifically in tumor cells may provide a promising therapeutic strategy for cancer. The best-characterized DRs and their cognate ligands thus far include TNFR1/TNF- α , Fas (CD95 or Apo1)/FasL (CD95L or Apo1L), DR4 and DR5/TRAIL (Apo2L), and Apo3

(DR3)/Apo3L (reviewed in Ref. 1). To explore the therapeutic potential of these ligands, we have examined thirteen genetically and biologically diverse human malignant glioma cell lines and primary cultures of human astrocytes for their sensitivity to these four ligands. Our results showed that TRAIL, but not TNF α , FasL, or Apo3L, can kill a large number of malignant glioma cell lines but not astrocytes. This has prompted us to address the regulatory mechanisms of TRAIL-induced apoptosis in malignant glioma cells.

TRAIL is a recently identified member of the TNF family that triggers rapid apoptosis in various types of tumor cells (2). TRAIL can interact with two DRs, DR4 (TRAIL-R1; Ref. 3) and DR5 (TRAIL-R2; Refs. 4 and 5), and two DcRs, DcR1 (TRID/TRAIL-R3; Refs. 4, 6, and 7) and DcR2 (TRUNDD/TRAIL-R4; Refs. 8 and 9). DR4 and DR5 contain an intracellular motif termed “death domain” that subsequently activates caspase-8 and the caspase cascade leading to apoptosis (3, 4, 10, 11). In contrast, DcR1 and DcR2 have either truncated or absent intracellular domains and are unable to transduce the death signal (4, 7, 9), suggesting that DcRs may compete for ligand-binding and act as antiapoptotic receptors.

The intracellular signaling that links TRAIL DRs and caspase-8 remains unclear. Studies of Fas and TNFR1 signaling pathways show that DRs bind, through their death domains, to the adapter protein FADD directly in Fas-induced apoptosis (12) and indirectly through another adapter protein named TRADD in TNFR1-induced apoptosis (13). FADD, in turn, interacts with caspase-8 through its DED (14, 15), leading to the assembly of a DISC (16). Caspase-8 oligomerization in the DISC drives its own activation through self-cleavage (17), subsequently activating downstream effector caspases leading to apoptosis (14, 15).

Studies of transfectants have produced controversial results as to the intracellular signaling in TRAIL-induced apoptosis. Some experiments demonstrate DR4 and DR5 binding to TRADD and FADD (5, 18), whereas others show no such interaction (3, 7). Several recent investigations of nontransfected cells, however, show consistently that DR4 and DR5 recruits FADD, but not TRADD, to activate caspase-8, a signaling pathway similar to that in Fas-mediated apoptosis (19–21). This agrees with a study that shows that cFLIP, an inhibitor of FLICE inhibits both FasL- and TRAIL-induced caspase-8 activation (22). cFLIP contains two DEDs and a FLICE/caspase-8-like domain that lacks catalytic activity (22). cFLIP interacts, through its DEDs, with FADD and caspase-8 to block caspase-8 binding to DISC, preventing DR-induced apoptosis (22).

Recently, another DED-containing protein, PED/PEA-15, has been identified (23, 24). PED/PEA-15 has a smaller molecular weight (M_r 15,000) than cFLIP (M_r 30,000–55,000) and does not possess a

domain; PED/PEA-15, phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes- M_r 15,000; z-VAD-fmk, carbobenzyloxy-Val-Ala-Asp(OMe)fluoromethyl ketone; z-DEVD-fmk, carbobenzyloxy-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)fluoromethyl ketone; z-IETD-fmk, carbobenzyloxy-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone; BDM, bisindolylmaleimide; TPA, 12-O-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; Act D, actinomycin D; CHX, cycloheximide; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C.

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³ The abbreviations used are: DR, death receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; DcR, decoy receptor; FasL, Fas ligand; FADD, Fas-associated death domain; TRADD, TNFR1-associated death domain; DISC, death-inducing signaling complex; FLICE, FADD-like IL-1 β -converting enzyme; cFLIP, cellular FLICE-like inhibitory protein; DED, death effector

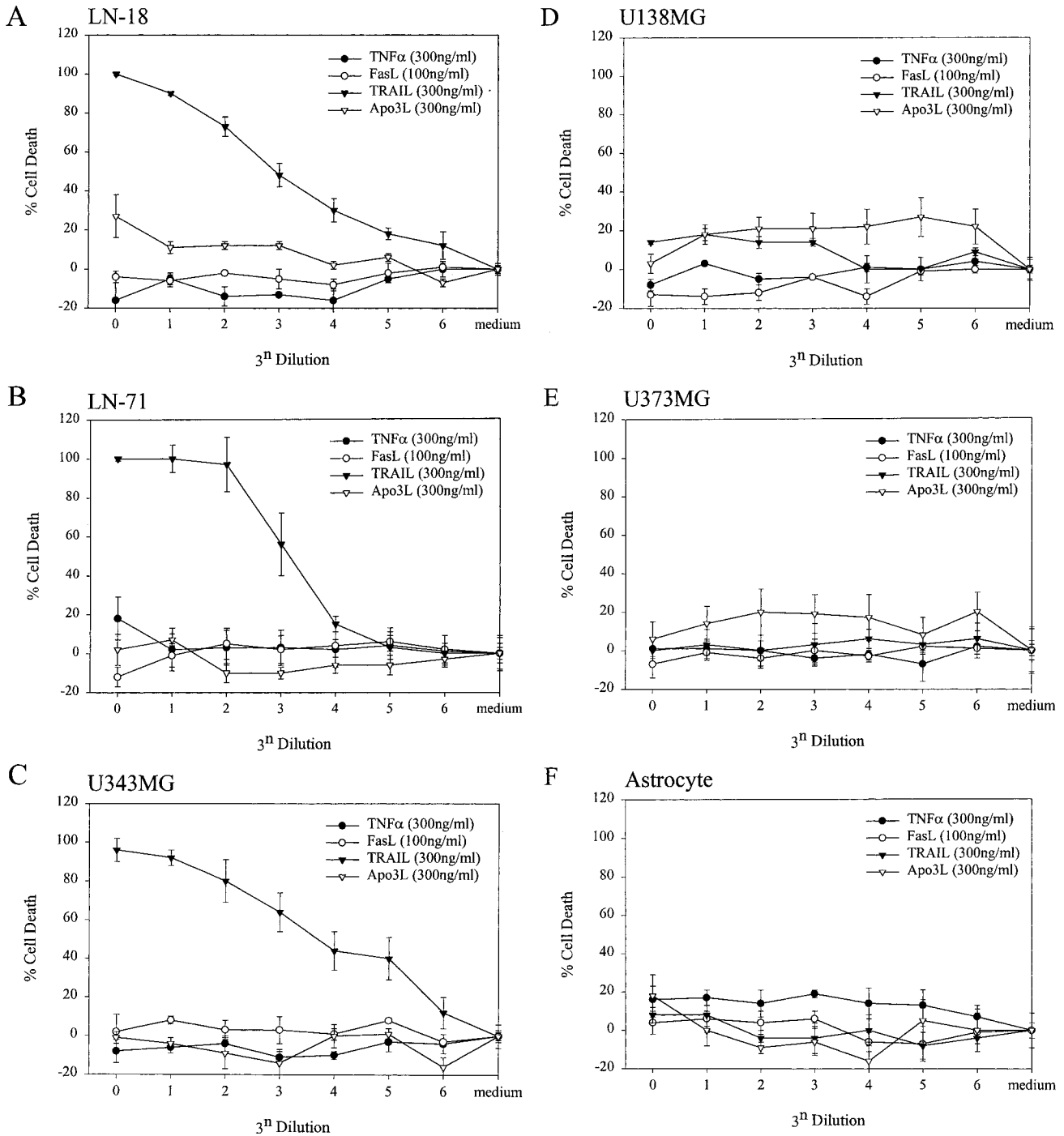


Fig. 1. Death ligand-induced cell death in five representative glioma cell lines (A–E) and normal astrocytes (F) as determined by crystal violet assay. The cells were seeded in 96-well plates at 2×10^4 cells/well and then treated with TNF α (starting at 300 ng/ml), FasL (100 ng/ml), TRAIL (300 ng/ml), and Apo3L (300 ng/ml) after 3-fold serial dilutions with growth medium as control. Each value represents the mean of triplicate samples and experiments were repeated three times.

caspase-like domain (23, 24). PED/PEA-15 is prominently expressed in astrocytes (23) and its expression protects astrocytes from TNF α -induced apoptosis (25). PED/PEA-15 inhibits TNFR1- and Fas-induced apoptosis through interruption of FADD-caspase-8 binding (26). In this study, we demonstrate that TRAIL induces apoptosis in 54% of glioma cell lines, whereas others remain resistant. Furthermore, we report the first direct evidence that intracellular PED/PEA-15, but not cFLIP, plays a crucial role in the negative regulation of TRAIL-induced apoptosis in malignant glioma cells.

MATERIALS AND METHODS

Materials. Recombinant human TRAIL, Apo3L, and TNF α were purchased from PeproTech, Inc. (Rocky Hill, NJ) and recombinant human FasL was from Calbiochem-Nova Biochem Corporation (San Diego, CA). The tetrapeptide caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, and z-IETD-fmk were purchased from Enzyme Systems Products (Livermore, CA), prepared as 20 mM stocks in DMSO and stored in aliquots at -20°C until further use. Act D and CHX were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada) and prepared as 1 mg/ml stock in DMSO and 10 mM stock

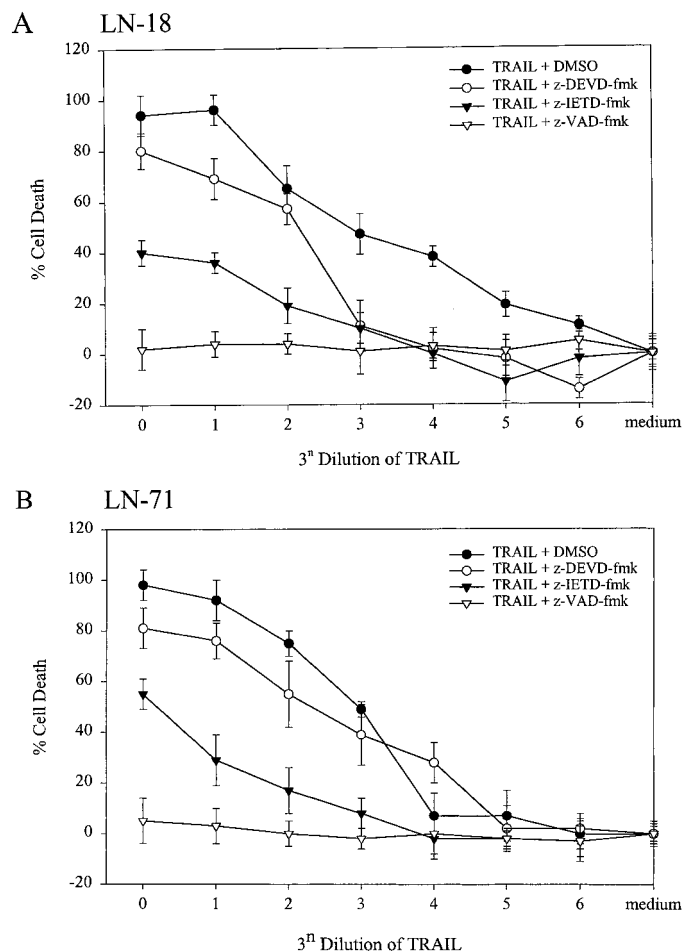


Fig. 2. Inhibition of TRAIL-induced cell death by caspase inhibitors as determined by crystal violet assay. TRAIL-sensitive cell lines LN-18 (A) and LN-71 (B) were seeded in 96-well plates at 2×10^4 cells/well and treated with 300ng/ml TRAIL and 3-fold serial dilutions in the presence of 20 μ M z-IETD-fmk, z-DEVD-fmk, z-VAD-fmk, or DMSO (vehicle) with growth medium as control. Each value represents the mean of triplicates and experiments were repeated three times.

in methanol, respectively. BDM and phorbol ester TPA were from Sigma (St. Louis, MO).

For PED antisense generation, PED cDNA (bp 1–65) was amplified by PCR using the primers 5'-GGCCGGTACCGCCAGAGCGCGGGGCA-GTGTG-3' and 5'-CGCGGATCCGTGCTCAATGATGGAGAGTTG-3' containing the *Kpn*I and *Hind*III cloning sites, respectively. The amplified fragment was subcloned in the *Kpn*I and *Hind*III sites of the pcDNA3 expression vector containing the neo^r marker. The pcDNA3-PED plasmid has been described previously (24).

Malignant Glioma and Melanoma Cell Lines and Normal Astrocyte Culture and Transfection. The human malignant glioma cell lines LN-18, LN-71, LN-215, LN-229, LN-Z308, and LN-751 were described previously (27) and kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). Other glioma cell lines, U87MG, U118MG, U138MG, U343MG, U373MG, and T98G were purchased from American Type Culture Collection. D247MG cells were kindly provided by Dr. D. Bigner (Duke University, Durham, NC). The human melanoma cell lines WM9, WM164, WM793, and WM3211 were kindly provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Burlington, Ontario, Canada). Three primary cultures of normal human fetal astrocytes were prepared and cultured as described previously (28), and the cells were evaluated at passage 3. Transfection of the pcDNA3 expression vector containing PED cDNA or antisense was accomplished using the lipofectamine method as described previously (24).

Cytotoxicity and Apoptosis. Cells were seeded in 96-well plates and then treated with TNF- α , FasL, TRAIL, or Apo3L. In some experiments, Act D (10

ng/ml), CHX (20 μ M), or synthetic caspase inhibitors (20 μ M z-DEVD-fmk, z-IETD-fmk, or z-VAD-fmk) were added together with TRAIL. Cytotoxicity was determined by the crystal violet assay as reported previously (29). Results were presented as the percentage cell death: $1 - (\text{optical density of cells treated} / \text{optical density at 550 nm of cells untreated}) \times 100$. Cell lines were considered as sensitive if 80–100% cell death was induced, partially resistant at 30–79% cell death, and resistant at <30% cell death induced by the highest concentration of the ligand. For cellular apoptosis, cells were treated with TRAIL in the presence or absence of CHX and examined under phase contrast light microscopy. For terminal deoxynucleotidyl transferase-mediated nick end labeling analysis of DNA fragmentation, cells in eight-well chamber slides were fixed with 4% paraformaldehyde in PBS, and DNA fragmentation was determined using cell death detection-POD kit (Roche Diagnostics, Laval, Quebec, Canada) according to the manufacturer's protocol.

RNAse Protection Assay. Total RNA was isolated from subconfluent cultures of the cell lines and astrocytes using TRIzol reagent (Life Technologies, Inc.). Equal amounts (10 μ g) of total RNA from each sample were hybridized with [α -³²P]UTP-labeled antisense RNA probes synthesized by T7 polymerase *in vitro*. RiboQuant multiprobe template sets were used according to manufacturer's protocol (BD Pharmingen, San Diego, CA).

Western Blot Analysis. Cells grown in a subconfluent culture were lysed in NP-40 lysis buffer [1%NP-40, 150 mM NaCl, and 50 mM Tris (pH8.0)] supplemented with fresh 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Cell lysates were centrifuged at $14,000 \times g$ to remove cellular debris, and protein concentrations were determined by Bio-Rad Dc Protein Assay kit (Bio-Rad Laboratories, CA). Equal amounts of the protein (20 μ g or 40 μ g) from each sample were separated through SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and blocked with 5% nonfat dry milk in TTBS [20 mM Tris-HCl (pH7.5), 500 mM NaCl, and 0.5% Tween 20] at room temperature for 1 h.

The membrane was incubated overnight at 4°C with a primary antibody diluted in ABS buffer solution (TTBS + 0.5% BSA). Primary rabbit antihuman antibodies to cFLIP (diluted at 1:1000), FADD (1:1000), DR4 (1:500), DR5 (1:1000), Dcr2 (1:1000), caspase-3 (1:5000), and extracellular signal-regulated kinases 1 and 2 (ERK1/2; 1:1000) were purchased from StressGen

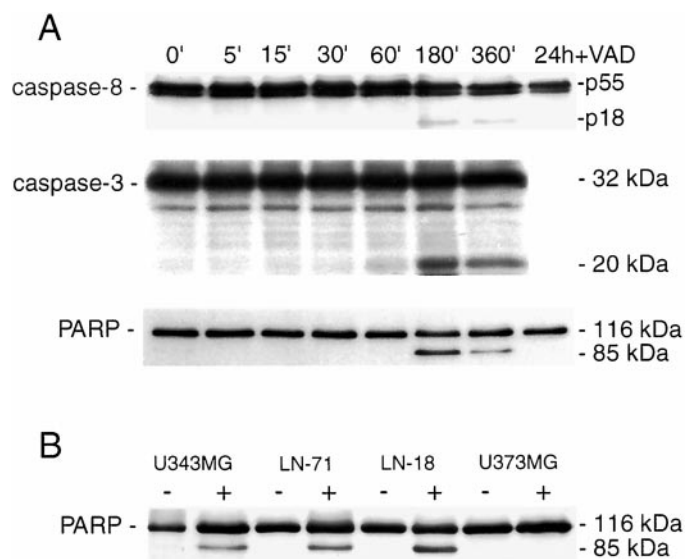


Fig. 3. Activation of caspase-8 and caspase-3 and PARP cleavage in TRAIL-induced glioma cell death. A, kinetic analysis of caspase-8 and caspase-3 activation and PARP cleavage. The TRAIL-sensitive line LN-18 was treated with 100 ng/ml TRAIL, alone or in combination with 20 μ M z-VAD-fmk (VAD) for the times indicated. Cell lysates were examined on Western blot. Cleavage of caspase-8 from the M_r 55,000 inactive form to the M_r 18,000 active subunit, caspase-3 from the M_r 32,000 (32-kDa) inactive form to the M_r 20,000 (20-kDa) active subunit, and PARP from a M_r 116,000 to an 85,000 (116–85 kDa) protein was detected 180 min after TRAIL was added to the culture. Caspase-8 inhibitor z-VAD-fmk prevented caspase-8 activation. B, cleavage of PARP in TRAIL-induced cell death. Three TRAIL-sensitive cell lines (U343MG, LN-71, and LN-18) and a resistant line (U373 MG) were treated with 100ng/ml TRAIL for 360 min, and the lysates were examined on Western blot. PARP cleavage was detected in all three sensitive glioma lines but not in the resistant line.

Biotechnology Corp. (Victoria, British Columbia, Canada). Rabbit anti-PED serum was described previously (24). Goat antihuman DcR1 (diluted at 1:200) was from R & D Systems (Minneapolis, MN). Mouse antihuman caspase-8 monoclonal antibody C15 was a kind gift from Dr. Marcus Peters (German Cancer Research Center, Heidelberg, Germany).

The membrane was washed and then incubated with peroxidase-conjugated goat antirabbit, rabbit antimouse, or rabbit antigoat antibody (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. The blots were washed and developed by chemiluminescence according to the manufacturer's protocol (NEN Life Science Products, Inc., Boston, MA). For quantification, the blots were subjected to Fluor-STM MultiImager analysis (Bio-Rad).

RESULTS

TRAIL Triggers Cell Death in Malignant Glioma Cells but not Normal Astrocytes. First, we examined the cytotoxic response of human malignant glioma cell lines and normal astrocytes to TNF- α , FasL, TRAIL, and Apo3L by crystal violet assay. All glioma cell lines and normal astrocytes were resistant to TNF- α , FasL, and Apo3L (Fig. 1). In contrast, seven of thirteen glioma cell lines were killed by TRAIL in a dose-dependent manner. Of the seven cell lines, three (LN-71, U343MG, LN-18) were sensitive (80–100% cell death) and four (T98G, U118MG, D247MG, and LN-229) were partially resistant (30–79% cell death). The remaining six cell lines (U87MG, U138MG, LN-215, LN-751, LN-Z308, and U373MG) and normal astrocytes were resistant to TRAIL-induced cell death at the maximum dose of 300 ng/ml (<30% cell death; Fig. 1).

TRAIL-induced Apoptosis in Glioma Cells Occurs through Activation of Caspase-8 and the Caspase Cascade. To determine whether TRAIL-induced cell death in sensitive glioma cell lines is mediated through the activation of intracellular caspases, we examined the ability of caspase inhibitors to prevent TRAIL-induced cell death. TRAIL sensitive lines (LN-71 and LN-18) were treated with 0–300 ng/ml TRAIL in the presence of z-IETD-fmk, z-DEVD-fmk, or z-VAD-fmk. The results (Fig. 2) showed that z-VAD-fmk completely protected the cell lines from TRAIL-induced cell death, whereas z-IETD-fmk and z-DEVD-fmk partially inhibited TRAIL-induced cell death. This implicates apical proapoptotic caspases in TRAIL-induced cell death.

To demonstrate caspase activation further, we examined TRAIL-sensitive cell lines for caspase-8 and caspase-3 activation and cleav-

age of the caspase substrate PARP. TRAIL-sensitive LN-18 cells were treated with TRAIL for various periods of time, and the cell lysates were examined by Western blot. Caspase-8 and caspase-3 activation and PARP cleavage were detected within 180 min after TRAIL treatment, but this was blocked by the presence of z-VAD-fmk (Fig. 3A). We further examined caspase-8 and caspase-3 activation and PARP cleavage in all three TRAIL-sensitive cell lines (U343MG, LN-71, and LN-18) in comparison with a TRAIL-resistant cell line (U373MG). PARP cleavage was detected in all of the sensitive cell lines, but not the resistant cell line (Fig. 3B). Similarly caspase-8 and caspase-3 activation were observed in the sensitive, but not the resistant, lines (data not shown). These results indicate a rapid activation of caspase-8 and the caspase cascade in TRAIL-induced glioma cell death.

Finally, we examined TRAIL-treated cell lines for cellular apoptosis and DNA fragmentation. TRAIL-sensitive LN-18 cells (Fig. 4A) were treated with TRAIL and cellular apoptosis marked by cell-surface blebbing was visualized morphologically under phase-contrast microscopy (Fig. 4B). TRAIL-induced cell surface blebbing was completely inhibited by z-VAD-fmk (Fig. 4C). DNA fragmentation was detected in the nuclei of glioma cells after TRAIL treatment (Fig. 4D). These results indicate that TRAIL induces apoptosis in glioma cells through activation of the caspase cascade.

DcR and Death Adaptor Proteins Expression Does Not Correlate with Glioma Sensitivity. TRAIL induced apoptosis in some glioma cell lines, but others remained resistant (Fig. 1). To identify molecules responsible for this resistance, we first examined glioma cell lines for their expression of all known TRAIL-Rs (signaling receptors DR4, DR5, and DcRs DcR1 and DcR2). RNase protection assays (Fig. 5A) and Western blots (Fig. 5B) established that DR5 mRNA and protein were detected at a high level in all of the cell lines tested. In contrast, DR4 mRNA and protein expression was limited to two sensitive cell lines, LN-71 and LN-18, one partially resistant cell line, D247MG, and one resistant cell line, U87MG (Fig. 5, A and B). These results indicate that DR5 is likely the major player in TRAIL-induced apoptosis of glioma cells, and that other molecules might modulate the sensitivity or resistance to TRAIL. DcRs might play an antiapoptotic role in glioma cells by competing for ligand binding. DcR1 mRNA (Fig. 5A) or proteins (not shown) were detected in

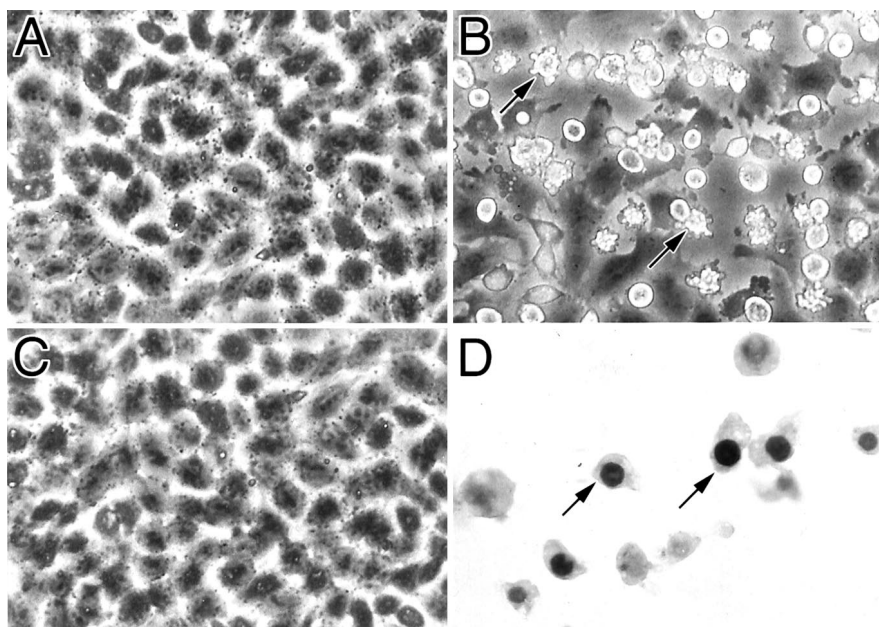


Fig. 4. Cellular apoptosis and DNA fragmentation in TRAIL-induced glioma cell death. LN-18 cells in 35-cm culture dishes (2×10^6 cells/dish; A–C) or in eight-well chamber slides (2×10^4 cells/well; D) were left untreated (A), treated with 100ng/ml TRAIL alone (B and D), or in combination with 20 μ M z-VAD-fmk (C) for 6 h. Cellular apoptosis marked by cell surface blebbing (arrows) was observed in the TRAIL-treated culture (B) but not in the z-VAD-fmk-treated culture (C). DNA fragmentation was seen in the nuclei (arrows) as detected by the cell death detection-POD kit (D).

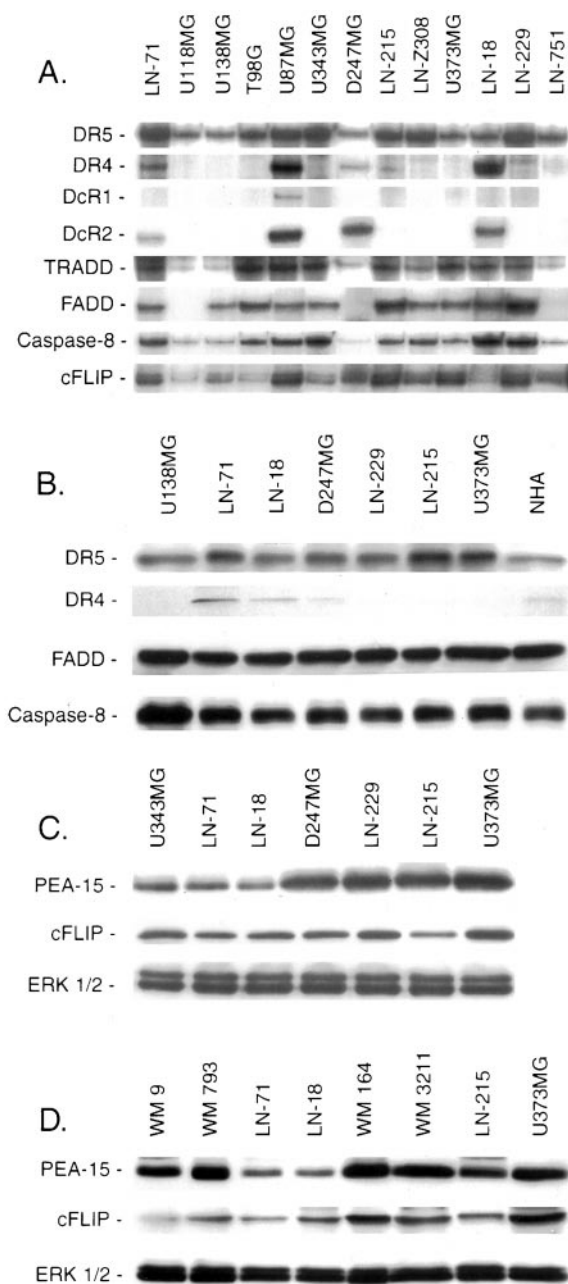


Fig. 5. Expression of TRAIL-signaling molecules in glioma and melanoma cells. *A*, mRNA expression in glioma cell lines. Thirteen glioma cell lines (listed at the top of the panel) were analyzed by RPA for their expression of TRAIL-signaling molecules (listed on the left side) as detailed in "Materials and Methods." *B*, DR5, DR4, FADD, and caspase-8 protein expression in glioma cells. Seven representative glioma cell lines (listed on the top of the panel) and normal human astrocytes (*NHA*) were subjected to Western blot analysis for their expression of these TRAIL apoptotic proteins. *C*, PED/PEA-15 and cFLIP expression in glioma cells. These seven glioma cell lines were analyzed further for their expression of the antiapoptotic protein PED/PEA-15 and cFLIP on Western blot. *D*, differential expression of PED/PEA-15 and cFLIP in glioma and melanoma cells. Both TRAIL-sensitive and -resistant glioma and melanoma cell lines were analyzed by Western blot for their expression of PED/PEA-15 and cFLIP protein. ERK1/2 was shown as a loading control in the Western blot. Western blot is described in detail in "Materials and Methods," and the data are interpreted in "Results."

resistant, partially resistant, or TRAIL-sensitive cell lines without clear correlation, suggesting that TRAIL-induced cell death is not regulated by the DcR expression level.

Subsequently, we examined glioma expression of intracellular adapter proteins (TRADD and FADD) and caspase-8 to determine whether the absence of these molecules may be responsible for the resistance. Transcripts for TRADD and caspase-8 were detected at

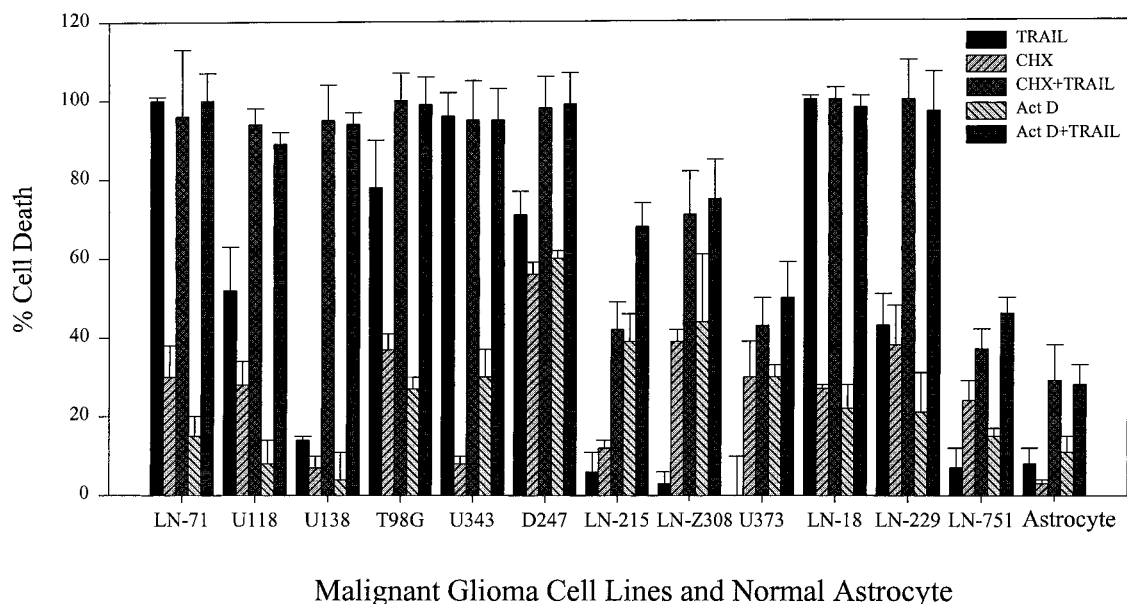
constant levels in all of the cell lines. FADD mRNA was found in all except a partially resistant cell line, U118MG (Fig. 5A). Western blot analysis further confirmed constant FADD and caspase-8 protein expression (Fig. 5B), indicating the presence of the components of the DR-dependent apoptotic machinery in both sensitive and resistant cell lines.

PED/PEA-15, but not cFLIP, Expression Correlates with TRAIL-resistant Glioma Cell Lines. The intracellular protein cFLIP has been reported to mediate TRAIL resistance in melanoma cell lines and transformed keratinocytes (10, 11). To determine whether cFLIP is also antiapoptotic in glioma cells, we examined glioma cell lines for their expression of cFLIP. To our surprise, cFLIP mRNA (Fig. 5A) and protein (Fig. 5C) were detected at a similar levels in all glioma cell lines, and expression did not correlate with TRAIL sensitivity. In searching for another DED-containing protein, we found that levels of PED/PEA-15 protein correlated with TRAIL resistance in glioma cells (Fig. 5C). Quantitative analysis showed that PED/PEA-15 expression was about two times higher in the resistant cell lines than that in the sensitive cell lines.

It is conceivable that PED/PEA-15 and cFLIP function as alternative antiapoptotic regulators in different tissue types. To test this hypothesis, we analyzed glioma and melanoma cell lines on the same Western blot to compare the level of expression of these two proteins in both tumor types. The four melanoma cell lines selected for the study included TRAIL-sensitive (WM 9 and WM 793) and resistant (WM 164 and WM 3211) cell lines (10). This analysis confirmed our earlier observation that PED/PEA-15, but not cFLIP, expression was lower in TRAIL-sensitive (LN-18 and LN-71) and higher in TRAIL-resistant (LN-215 and U373MG) glioma cell lines (Fig. 5D). In contrast, cFLIP, but not PED/PEA-15, protein was expressed at slightly lower levels in sensitive melanoma cells (Fig. 5D) consistent with previous reports (10).

Protein Synthesis Inhibitors Increase Glioma Sensitivity to TRAIL through Reduction of Apoptosis Inhibitor PED/PEA-15. Protein synthesis inhibitors were reported to increase sensitivity to death ligand-induced apoptosis, presumably by reducing the expression of antiapoptotic proteins with rapid turnover (30). To further confirm the inhibitory role of PED/PEA-15 proteins in TRAIL-induced apoptosis, we examined the glioma cell lines to see if protein synthesis inhibitors enhance their sensitivity to TRAIL through inhibition of PED/PEA-15 production. Normal astrocytes and twelve glioma cell lines were treated with TRAIL, Act D or CHX alone or in combinations (Fig. 6). All partially resistant lines (T98G, U118MG, D247MG, and LN-229) and one resistant line (U138MG) became sensitive to TRAIL in the presence of Act D or CHX (Fig. 6). However, normal astrocytes and four resistant cell lines (LN-215, LN-308, U373MG, and LN-751) remained largely resistant. Caspase-3 activation and PARP cleavage (Fig. 7), as well as cellular apoptosis and DNA fragmentation (Fig. 8), were demonstrated in a convertible resistant cell line (U138MG), but not in a resistant line (U373MG) after TRAIL treatment with or without CHX and z-VAD-fmk treatments. These results suggest that CHX treatment is necessary to restore TRAIL sensitivity in resistant cells but is not sufficient to induce apoptosis *per se*.

The fact that protein synthesis inhibitors restore TRAIL-induced apoptosis in some resistant cell lines suggests that the death machinery is intact and functional in these cells, yet is under negative regulation by antiapoptotic proteins with rapid turnover. cFLIP and PED/PEA-15 are candidates for such inhibitors (22–24). It was reported that that cFLIP levels are rapidly reduced after protein synthesis inhibitor treatment in melanoma cells (10). To test this possibility in glioma cells, we examined PED/PEA-15 and cFLIP protein expression in LN-18 (sensitive, used as control), U138MG (convertible



Malignant Glioma Cell Lines and Normal Astrocyte

Fig. 6. Effects of protein synthesis inhibitors on glioma sensitivity to TRAIL. Cells seeded in 96-well plates (2×10^4 cells/well) were treated with TRAIL (100 ng/ml), 10 ng/ml Act D, or 20 μ M CHX, alone or in combination, for 24 h. The cell death was determined by crystal violet assay. Each value represents the mean \pm SD of triplicate samples and experiments were repeated three times.

resistant), and U373MG (resistant) cell lines. Cells were treated with 100 ng/ml TRAIL alone (LN-18) or in combination with 20 μ M CHX (U138MG and U373MG). PED/PEA-15 protein levels were decreased in LN-18 (Fig. 9A) and U138MG (Fig. 9B) but not in the most resistant line, U373MG (Fig. 9C). In contrast, cFLIP protein levels were unaffected in all of the three lines, suggesting that the CHX concentrations used were insufficient to prevent cFLIP synthesis, or that cFLIP is very stable in glioma cells. The artificial down-regulation of PED/PEA-15 with concentrations of protein synthesis inhibitors that do not affect cFLIP levels over a 6-h period are sufficient to restore TRAIL sensitivity in “convertible” resistant cell lines, indicating that PED/PEA-15, but not cFLIP, may play a crucial role in the negative regulation of TRAIL-induced apoptosis in glioma cells.

PED/PEA-15 Antisense Transfection Converts TRAIL-resistant Lines to Sensitive Lines. To define further the function of PED/PEA-15 in determining TRAIL-resistance in glioma cells, we transiently transfected sense or antisense PED/PEA-15 cDNA in sensitive and resistant cells (Fig. 10). TRAIL exposure of untransfected or vector control transfected LN-18 cells caused a 2-fold increase in DNA fragmentation (Fig. 10A). In contrast, transfection of the cells with PED/PEA-15 sense cDNA resulted in a 10-fold overexpression of PED/PEA-15 protein and completely blocked the TRAIL-induced DNA fragmentation (Fig. 10A). Similar effects are observed in another TRAIL-sensitive line U343MG (data not shown). Transient expression of PED/PEA-15 antisense cDNA reduced PED/PEA-15 levels in U373MG cells by >70% and caused a 3-fold increase in TRAIL-induced DNA fragmentation as compared with controls (parental cells and vector control transfectants). A similar effect was observed upon transfection of PED/PEA-15 antisense in another TRAIL-resistant line, U138MG (data not shown). Collectively, these results indicate that PED/PEA-15 plays a crucial role in the negative regulation of TRAIL-induced apoptosis in glioma cells.

PED/PEA-15 is a substrate of PKC *in vitro* and in intact cells (24). It was shown that inhibition of PKC blocked the PED/PEA-15 inhibition of TNF α -induced apoptosis, implying the importance of phosphorylation by PKC in PED/PEA-15 function (26). To verify whether phosphorylation by PKC is important for PED/PEA-15 function in TRAIL-induced apoptosis, we inhibited the activity of PKC in

TRAIL-resistant U373MG cells by either down-regulating PKC with the phorbol ester TPA or by blocking PKC with BDM (Fig. 10C). Both treatments alone did not significantly affect DNA fragmentation in unstimulated cells. The combination of TPA or BDM with TRAIL induced apoptosis, suggesting that resistance to TRAIL in U373MG cells is dependent on PKC-mediated phosphorylation.

DISCUSSION

Malignant gliomas of astrocytic origin are the most common and the most aggressive human brain tumors. Advances in standard treatments for these tumors, such as surgery, radiotherapy, and chemotherapy, have not significantly improved patient survival. Recent studies have demonstrated that targeting TNF superfamily DRs by their cognate ligands is a promising strategy for the treatment of cancers (31, 32). In the present study, we examined a large panel of glioma cell lines for their susceptibility to death ligands and demon-

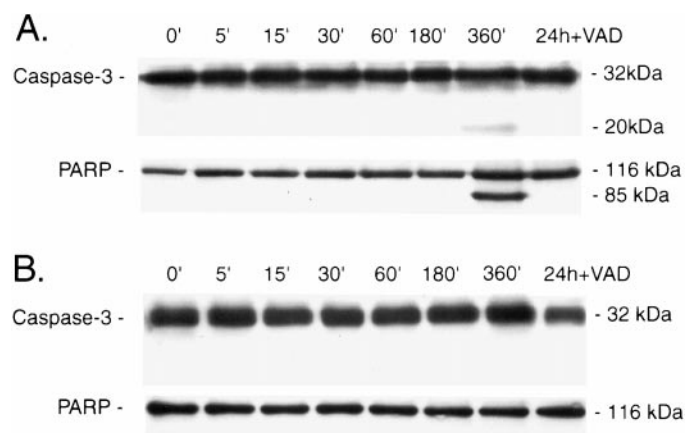


Fig. 7. Caspase-3 and PARP cleavage in the convertible TRAIL-resistant cell line. U138MG (A) and U373MG (B) cells were treated with 100 ng/ml TRAIL together with 20 μ M CHX or with 20 μ M z-VAD-fmk (VAD) for the times indicated. Caspase-3 and PARP cleavage was detected on Western blot in the convertible resistant line U138MG cells (A) but not in U373MG (B). Caspase inhibitor z-VAD-fmk prevented the caspase-3 and PARP cleavage in U138MG cells (A).

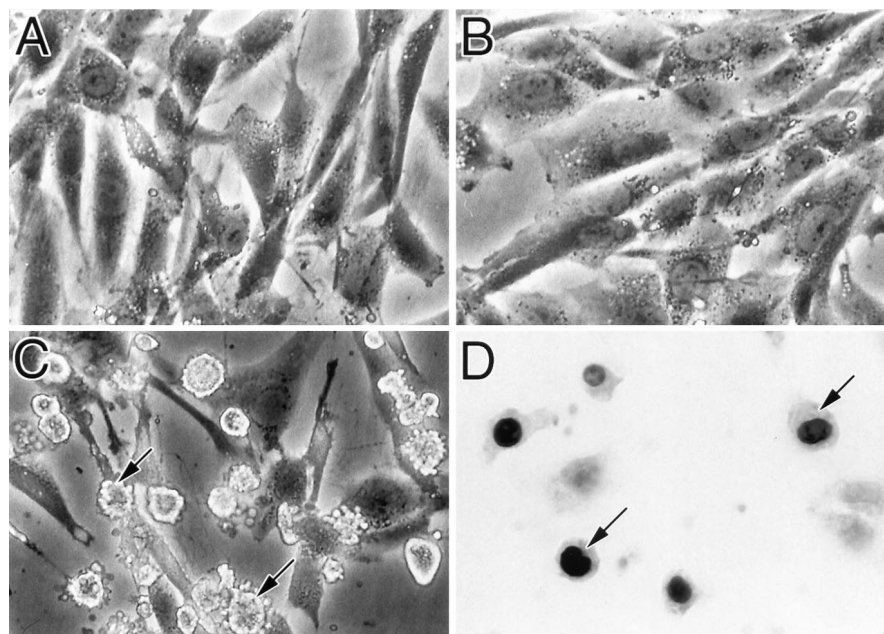


Fig. 8. Cellular apoptosis and DNA fragmentation in the convertible TRAIL-resistant cells. U138MG cells grown in 35-cm culture dishes (2×10^6 cells/dish; A–C) or eight-well chamber slides (2×10^4 cells/well) were left untreated (A), treated with 100 ng/ml TRAIL alone (B), or in combination with 20 μ M CHX (C and D) for 6 h. Cellular apoptosis marked by cell-surface blebbing (C; arrows) and DNA fragmentation in nuclei (D; arrows) were seen only in the culture treated with TRAIL and CHX.

strated that, of four well-characterized soluble death ligands (TNF α , FasL, Apo3L, and TRAIL), TRAIL killed the majority (54%) of human malignant glioma cells with medium to high efficiency. Fas-mediated apoptosis of glioma cells using anti-Fas antibodies or membrane bound FasL has been reported previously (33, 34). Soluble FasL apoptotic-inducing capacity is reduced compared with membrane-bound FasL (35). The use of soluble FasL in this study and the high constitutive levels of cFLIP in the cells might explain why little Fas-mediated cell death was observed.

TRAIL triggers apoptosis in tumor cells by binding to its cognate DRs on the cellular surface (2). Malignant glioma cells express high levels of TRAIL DRs and appear to be susceptible to TRAIL-induced cell death (36, 37). Of two TRAIL DRs (DR4 and DR5), we found that malignant glioma cells primarily express DR5, suggesting that TRAIL-induced apoptosis may occur primarily through DR5 activation. In support of this hypothesis, a recent study revealed that DR5 expression was increased by DNA-damaging chemotherapeutic drugs, thus enhancing TRAIL killing of glioma cells (38). We further demonstrated that TRAIL killing of glioma cells is characterized by caspase-8 and caspase-3 activation, PARP cleavage, DNA fragmentation, and the morphological features of cellular apoptosis, indicating the presence of a caspase pathway in TRAIL-induced apoptosis that is similar to that observed with other TNF family members (1).

Our study also demonstrates that the efficiency of TRAIL-induced apoptosis in malignant gliomas is variable, and many glioma cell lines are either partially or completely resistant to this ligand. Expression of DcR1 and DcR2 has been proposed to explain resistance to DR-mediated apoptosis (4, 6, 8, 9). DcR1 and DcR2 expression was limited in the malignant glioma cells analyzed here and did not correlate with susceptibility to TRAIL, consistent with findings in melanoma cells and transformed keratinocytes (10, 11).

Here, we showed that the TRAIL-resistant phenotype of some glioma cells can be converted to sensitive by treatment with protein synthesis inhibitors. This suggests the presence in the cells of short-lived apoptosis inhibitors. The demonstration of caspase-8 activation in these cells indicates that regulation of apoptosis may occur in between the DRs and caspase-8. Apoptosis inhibitor cFLIP is a prime candidate for this intracellular regulation because it possesses a DED through which it interrupts FADD and caspase-8 binding, preventing

DISC formation and apoptosis (22). cFLIP expression in melanoma cells correlated with their sensitivity to TRAIL, as reported in one study (10) but not another (39); cFLIP expression in glioma cells did not correlate with sensitivity to TRAIL in our study. Furthermore, protein synthesis inhibitors did not decrease cFLIP expression at concentrations enhancing TRAIL-induced apoptosis. These results

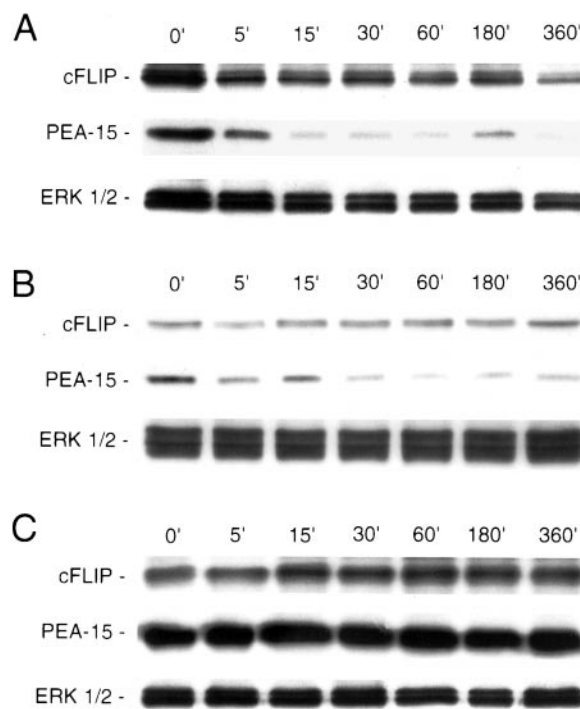


Fig. 9. Differential expression of PED/PEA-15 and cFLIP in glioma cell lines. Sensitive line LN-18 was treated with 100 ng/ml TRAIL alone (A) whereas the convertible resistant line U138 MG (B) and the resistant line U373MG (C) were treated with 100 ng/ml TRAIL and 20 μ M CHX for the times indicated. Equal amounts of proteins (40 μ g/lane) prepared from each of these cultures were loaded and then analyzed by Western blot. Levels of ERK1/2 were used as control for even protein loading. Markedly decreased PED/PEA-15 protein levels were seen in LN-18 (A) and U138MG (B) but not in U373MG (C). In contrast, cFLIP protein expression levels were constant in all of the three cell lines.

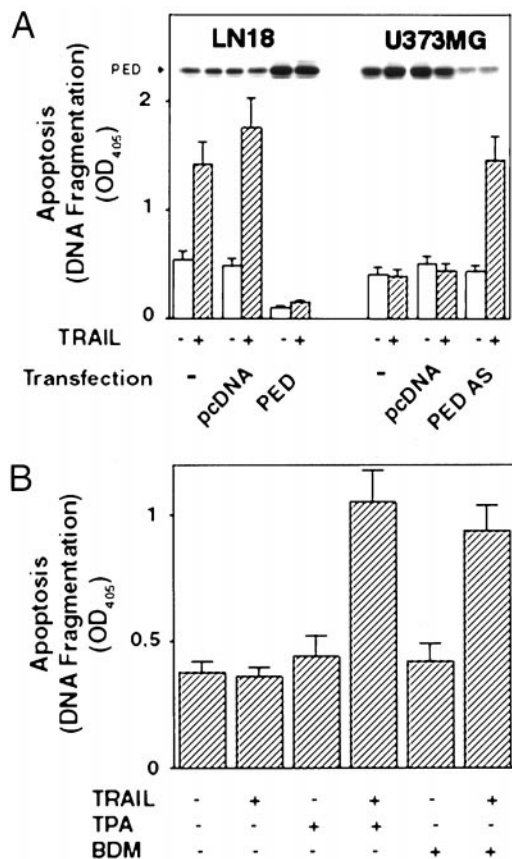


Fig. 10. PED/PEA-15 function in resistance to TRAIL. A, PED/PEA-15 cDNA and antisense transfection. TRAIL-sensitive LN-18 and TRAIL-resistant U373MG cells were transiently transfected with PED/PEA-15 cDNA and antisense (*PED AS*), respectively or with the plasmid DNA (*pcDNA*) alone, as indicated. The cells were incubated further with 100 ng/ml TRAIL, and apoptosis was quantitated by evaluating the level of DNA fragmentation using the Cell Death Detection-POD kit. Part of the cells were solubilized and Western blotted with PED/PEA-15 antibodies (*insets*). B, U373MG cells were preincubated with 1 mM phorbol ester TPA for 24 h or with 100 nM BDM for 45 min and subsequently exposed to TRAIL and assayed for apoptosis. *Bars*, \pm SD of duplicate determinations in three independent experiments.

suggest that cFLIP is unlikely to be directly involved in regulating TRAIL-induced apoptosis in glioma cells.

Our next candidate was PED/PEA-15, a recently cloned DED-containing protein that is highly expressed in astrocytes (23, 24). PED/PEA-15 was shown to interfere with caspase-8 and FADD binding, preventing DISC formation and subsequent apoptosis initiated by FasL and TNF- α (26). We now provide several lines of evidence that PED/PEA-15 inhibits TRAIL-induced apoptosis in glioma cells. Western blot analysis of glioma cell lines showed a clear correlation between glioma expression of PED/PEA-15 protein and sensitivity to TRAIL-induced apoptosis. Concentrations of protein synthesis inhibitors that reduced PED protein expression also enhanced TRAIL-induced apoptosis in resistant cell lines. Transfection of PED antisense in TRAIL-resistant cell lines converted these lines into sensitive lines, whereas transfection of PED cDNA in TRAIL-sensitive cell lines rendered the cells resistant. Inhibition of PKC activity restored TRAIL-induced apoptosis in resistant cells, demonstrating that induction of apoptosis in TRAIL-resistant glioma cells is prevented by PKC phosphorylation. The critical PKC substrate is likely PED/PEA-15 because its antiapoptotic activity is PKC-dependent (24).

Our studies have shown that TRAIL induces apoptosis in a majority of glioma cell lines, but not in primary cultures of normal astrocytes, thus providing a therapeutic potential for malignant gliomas. Systemic

TRAIL administration has been shown to suppress the growth of a number of tumor types in animals, with little toxicity (31, 32). However, a recent study has revealed species-related differences in the sensitivity of hepatocytes to TRAIL, *i.e.*, induction of apoptosis in cultured human hepatocytes but not in hepatocytes isolated from other species (40). This finding has important safety implications for systemic, but not local, TRAIL administration in humans. Although of significant clinical concern for systemic cancers, malignant gliomas rarely metastasize, and intratumoral administration of TRAIL is a potential treatment option. This will only be possible when a comprehensive study has evaluated the sensitivity of the diverse cell populations in the human brain to TRAIL. The finding that glioma cell lines can be sensitive, partially or completely resistant to TRAIL suggests the presence of more complex regulatory mechanisms in TRAIL-induced apoptosis. Understanding these mechanisms, by which some glioma cells and normal brain cells maintain resistance to TRAIL, may help to optimize TRAIL therapy for malignant gliomas as well as for other cancers.

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