RESEARCH ARTICLE

TRAIL Triggers Apoptosis in Human Malignant Glioma Cells Through Extrinsic and Intrinsic Pathways

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Many malignant glioma cells express death receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), yet some of these cells are resistant to TRAIL. Here, we examined signaling events in TRAIL-induced apoptosis and searched for therapeutic agents that could overcome TRAIL resistance in glioma cells. TRAIL induced apoptosis through death receptor 5 (DR5) and was mediated by caspase-8-initiated extrinsic and intrinsic mitochondrial pathways in sensitive glioma cell lines. TRAIL also triggered apoptosis in resistant glioma cell lines through the same pathways, but only if the cells were pretreated with chemotherapeutic agents, cisplatin, camptothecin and etoposide. Previous studies suggested that this was due to an increase in DR5 expression in wild-type *TP53* cells, but this mechanism did not account for cells with mutant *TP53*. Here, we show that a more general effect of these agents is to downregulate caspase-8 inhibitor c-FLIP$_S$ (the short form of cellular *F*as-associated death domain-*l*ike interleukin-1-converting enzyme-*i*nhibitory *p*rotein) and up-regulate Bak, a pro-apoptotic Bcl-2 family member, independently of cell's *TP53* status. Furthermore, we showed that TRAIL alone or in combination with chemotherapeutic agents, induced apoptosis in primary tumor cultures from patients with malignant gliomas, reinforcing the potential of TRAIL as an effective therapeutic agent for malignant gliomas.

Brain Pathol 2003;13:539-553.

Introduction

Death ligands of the tumor necrosis factor (TNF) family such as TNFα and Fas ligand (FasL, CD95L) can trigger apoptosis in solid tumors, but their potential clinical use has been very limited due to severe toxicity to normal tissues (42, 51). TNF-related apoptosis-inducing ligand (TRAIL) is a recently identified member of the TNF family, but unlike TNFα and FasL that are only expressed in activated cells, TRAIL is found in normal tissues (46, 68) and is involved in tumor surveillance (62). In vitro and in vivo studies have shown that TRAIL induces apoptosis in tumor cells but not in most normal cells and may prove to be a candidate for cancer therapy (3, 28, 66). To explore this therapeutic potential in malignant gliomas, we examined a large panel of human glioma cell lines (23) and showed that some cell lines are sensitive whereas others are resistant to TRAIL killing (19). These findings have prompted us to investigate the signal transduction events in TRAIL-induced apoptosis in glioma cells to define the molecular mechanisms that control glioma cells resistance.

TRAIL induces apoptosis through 2 death receptors, DR4 (TRAIL-R1) (43, 44, 52) and DR5 (TRAIL-R2) (6, 53, 55, 65, 70). DR4 and DR5 have extracellular, cysteine-rich pseudorepeats for TRAIL-binding and intracellular death domains that recruit the cytoplasmic adapter, Fas-associated death domain (FADD) (4, 25, 26, 38). FADD has a carboxy-terminal death domain and an amino-terminal death effector domain through which it recruits caspase-8, resulting in the assembly of a death-inducing signaling complex (DISC) (5, 24). Within the DISC, caspase-8 is able to complete a 2-step, autoproteolytic cleavage (39) to release its active subunits into the cytosol where they initiate apoptosis through cleavage of downstream caspases (71). Recently, another death effector domain-containing protein was reported and termed c-FLIP for cellular FADD-like IL-1β-converting enzyme (FLICE)-inhibitory protein (22). c-FLIP is expressed as 2 isoforms (22, 50, 56): the short form (c-FLIP$_S$, M_r ~28 kDa), contains 2 death effector domains and the long form (c-FLIP$_L$, M_r ~55 kDa) has 2 death effector domains and a caspase-like domain that lacks catalytic activity. Both the short and long forms of c-FLIP are recruited to the Fas- and TRAIL-induced DISC to inhibit caspase-8 cleavage and thus prevent apoptosis (71, 72).

There are 2 major signaling pathways that control apoptosis initiation: the extrinsic pathway through

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death receptors (2) and the intrinsic pathway via mitochondria (15). There is increasing evidence for cross-talk between extrinsic and intrinsic pathways in TRAIL-induced apoptosis. Once caspase-8 is activated in the DISC, it can either directly cleave caspase-3 to activate extrinsic pathways or cleave the Bcl-2 inhibitory BH3-domain-containing protein (Bid) to activate the intrinsic pathway (17). Truncated Bid (tBid) translocates to the mitochondrial membrane and induces loss of mitochondrial transmembrane potential and release of apoptotic factors such as cytochrome *c* (32, 34), apoptosis-inducing factor (AIF) (61) and Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis binding protein [IAP] with low pI) (9, 63). In the cytosol, cytochrome *c* binds to Apaf1 to recruit dATP and caspase-9 to form an apoptosome where caspase-9 is activated, and in turn cleaves caspase-3, leading to apoptosis (31, 58, 74). Smac/DIABLO, once released from mitochondria, interacts with X-linked inhibitor of apoptosis protein (XIAP), to release XIAP inhibition of caspase-3 and caspase-9, thus promoting apoptosis (7, 59).

The mitochondrial membrane potential is controlled by Bcl-2 family members (8). Anti-apoptotic members, Bcl-2 and Bcl-X_L, inhibit the release of mitochondrial apoptotic factors whereas pro-apoptotic members, Bax and Bak, trigger their release. Bak resides on the mitochondrial membrane (8) while Bax translocates from cytosol to mitochondria through interaction with tBid (69). tBid induces Bax and Bak oligomerization and thus loss of mitochondrial membrane potential (16, 67). Bcl-2 and Bcl-X_L are bound to the outer mitochondrial membrane and interact with Bax and Bak to maintain the mitochondrial membrane potential (8). Overexpression of Bcl-2 or Bcl-X_L blocks TRAIL-induced apoptosis (20, 37, 41, 47, 60), whereas deletion of Bax from TRAIL-sensitive cells results in the cell resistance to TRAIL (29).

Conventional chemotherapeutic drugs, camptothecin (CPT), cisplatin and etoposide (VP16), have been reported to enhance TRAIL-induced apoptosis in various tumor cells (11, 12, 14, 27, 40), but the molecular mechanisms of their action remain controversial. Some groups reported that cisplatin and VP16 upregulate DR4 and DR5 (11, 14, 40), whereas others showed that cisplatin increases mitochondrial release of cytochrome *c* to sensitize resistant cancer cells to TRAIL (37, 60). In this study, we first demonstrate that TRAIL-induced apoptosis in malignant glioma cells occurs through both extrinsic and intrinsic pathways. We then show that CPT and cisplatin down-regulate c-FLIP to release its inhibition of caspase-8 cleavage in the DISC and that they up-regulate Bak to promote mitochondrial release of apoptotic factors, thus enhancing TRAIL-induced apoptosis in malignant glioma cells.

Materials and Methods

Materials. Recombinant soluble form of human TRAIL (amino acids 114-281) was a kind gift from PeproTech, Inc. (Rocky Hill, NJ). Camptothecin (CPT), cisplatin and etoposide (VP16) (Sigma-Aldrich Canada Ltd, Oakville, Ontario) were prepared as 100 mg/ml stock in DMSO with the final concentration of DMSO not exceeding 0.1% (v/v). The mouse monoclonal antibodies used in the study included c-FLIP NF6 clone (kind gift from Dr Peter Krammer, German Cancer Research Center, Heidelberg) (50), FADD, cytochrome *c*, and XIAP (Transduction Laboratories, Lexington, KY), caspase-8 (Medical & Biological Laboratories, Nagoya, Japan) and cytochrome *c* oxidase (COX) IV (Molecular Probes, Eugene, Ore). Rabbit polyclonal antibodies included anti-human caspase-3, DFF45 and ERK1/2 (StressGen, Victoria, British Columbia), Bid (Biosource International, Inc., Camarillo, Calif), Bak (Upstate Biotechnology, Lake Placid, NY), Bcl-2, Bcl-X_L, Bcl-X_S, Bax (Santa Cruz Biotechnology, Santa Cruz, Calif), Smac and AIF (Biomol, Plymouth Meeting, Pa). Goat antibody to β-actin was purchased from Santa Cruz Biotechnology. Phycoerythrin-conjugated anti-human DR4 and DR5 mouse IgG_1 were purchased from *e*Bioscience (San Diego, Calif) and phycoerythrin-conjugated IgG_1 was from BD PharMingen (San Diego, Calif). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG2a, IgG2b and rabbit anti-goat IgG were from Southern Biotech (Birmingham, Ala); HRP-conjugated goat anti-rabbit antibody was from Jackson Immunoresearch Laboratories (West Grove, Pa). Mouse IgG1, protease inhibitor mixture, Triton X-100, Tween-20, and other chemicals of analytical grade were purchased from Sigma-Aldrich.

Cell culture, Cell death and apoptosis. The human malignant glioma cell lines have been previously reported (23). The cell lines were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (Life Technologies, Inc.) in a humidified 5.0% CO_2 and 37°C incubator. Early passages of primary brain tumor cultures were established from fresh operative tumor samples and cultured in DMEM/F-12 (Invitrogen) supplemented with 10% FBS, 1% non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% antibiotics,

as previously reported (45). For cell death assay, the cells were plated at 3×10^4 cells/well in 96-well plates and incubated overnight. The cells were then treated for 16 hours with human recombinant TRAIL, CPT, cisplatin and VP16, alone or in their combination, as indicated in the Results. Cell death was assessed by the crystal violet assay and results were presented as the percentage cell death: 1- (optical density of cells treated/optical density at 550 nm of cells untreated) $\times$ 100 (19). For cellular apoptosis, cells were treated with TRAIL, alone or in combination with chemotherapeutic drug and examined under phase contrast light microscopy (19). For cleavage of caspases and DFF45, subconfluent cells were treated with TRAIL, alone or in combination with cisplatin, CPT or VP16 for 16 hours. The cells were lysed and subjected to Western blotting (71, 72).

Flow cytometry analysis. Cell surface expression of DR4 and DR5 was measured by flow cytometry. In brief, 0.1 μg/ml of phycoerythrin-conjugated anti-human DR4 and DR5 (mouse IgG_1) or mouse IgG_1 (a negative control) were added to the 10^6 cells in 200 μl of immunofluorescence (IF) buffer (PBS containing 2% FBS and 0.02% sodium azide [Sigma-Aldrich]). After one hour of incubation in the dark at 4°C, the cells were washed with IF buffer and then dispersed 500 μl PBS. For all tested cell samples, 10 000 cells were analyzed using a Becton and Dickinson FACScan™ (Mountain View, Calif), and the data were processed by using Cell Quest™ software (Becton and Dickinson).

Subcellular fractionation. Subconfluent cells were treated with TRAIL or chemotherapeutic agents as indicated in the Results. The cells were harvested and the cell pellet was suspended in 5 volumes of isotonic buffer (10 mM HEPES-KOH [pH 7.5], 210 mM mannitol, 70 mM sucrose, 1 mM Na-EDTA, and 1 mM Na-EGTA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture. The cells were incubated on ice for 15 minutes and passed through a 22-gauge needle 15 times. After centrifugation twice at 700g for 10 minutes at 4°C, the supernatant was collected and centrifuged at 13 000g for 10 minutes at 4°C. The resulting mitochondrial pellets were suspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton-X 100, 1 mM PMSF, and 0.2% protease inhibitor cocktail). The supernatants of the 13 000g spin were further centrifuged at 100 000g for one hour at 4°C, and the resulting supernatants were designated as the S-100 cytosolic fractions. The protein concentrations in cytosolic and mitochondrial fractions were determined by the Bradford assay (Bio-Rad) and equal amounts of mitochondrial and cytosolic proteins were subjected to Western blot analysis using cytosol-specific β-actin antibody and mitochondrial specific COX IV antibody as loading controls.

Western blot. Subconfluent cells were either treated or untreated and then lysed in a cold lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1% protease inhibitor mixture and 1 mM PMSF). The cell lysates were centrifuged for 15 minutes at 18 000g at 4°C, supernatants were collected and protein concentrations in the supernatant was determined by a Bradford assay. Cell lysates (75 μg), and cytosolic and mitochondrial fractions (25 μg) were subjected to SDS-polyacrylamide electrophoresis (12.5-15%) and transferred to nitrocellulose membranes. The membranes were blocked in TBST (5% milk in Tris-buffered saline with 0.05% Tween-20), then blotted overnight with various primary antibodies as indicated in the Results. The membranes were washed and incubated for one hour with HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody. The membranes were washed and developed by chemiluminescence (Amersham Biosciences).

Northern blot. Two 11-cm diameter tissue culture dishes with LN382 cells were grown at 37°C until 90% confluent as recommended (23). Twenty-four hours before total RNA preparation with Trizol (Gibco-BRL) one flask was transferred to 34°C. Northern blot was prepared with 15 μg total RNA and hybridized subsequently with p21, p53 and GAPDH cDNA probes as previously described (21).

Results

TRAIL induces apoptosis in glioma cells through extrinsic and intrinsic pathways. We have previously found that about half of human malignant glioma cell lines are sensitive to TRAIL (19). In this study, we further examined these cells to determine if TRAIL-induced apoptosis occurs through the extrinsic, intrinsic or both pathways. First, we examined cell surface expression of TRAIL receptors in TRAIL-sensitive cell lines U343MG and LN71. Flow cytometry analysis showed that both expressed DR5, but not DR4 (Figure 1A). We then treated the cells with TRAIL and showed a dose-dependent cell killing with effective doses ranging from 33 to 300 ng/ml, as determined by crystal violet assay (Figure 1B). Phase-contrast microscopy evi-

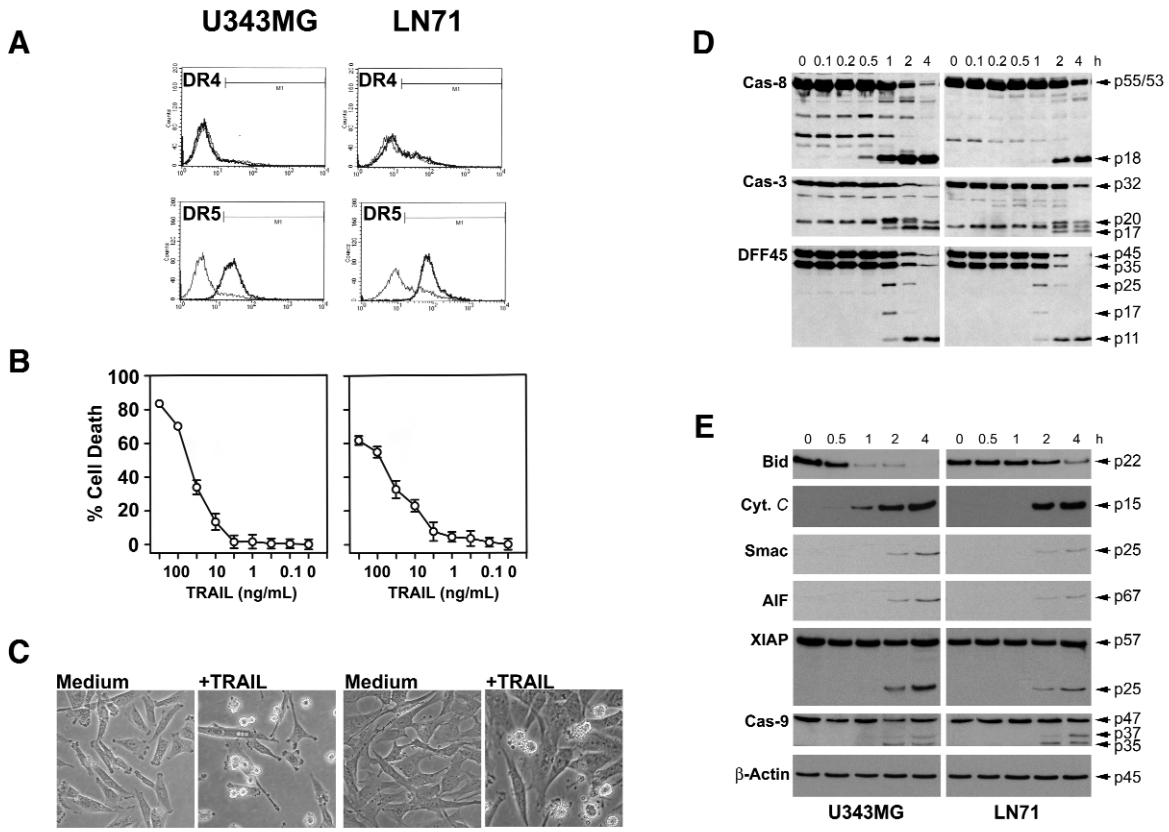


Figure 1. TRAIL induces apoptosis through DR5-mediated caspase-8-initiated extrinsic and intrinsic pathways in TRAIL-sensitive glioma cells (U343MG and LN71). **A.** Detection of cell surface expression of DR4 and DR5 by flow cytometry. **B.** Detection of TRAIL-induced cell death by crystal violet assay. Cells were grown in 96-well plates (3×10^4 cells/well) overnight and then treated with various doses of TRAIL for 16 hours. Data is mean ± standard error mean (SEM, n = 6). **C.** Detection of cellular apoptosis under phase contrast microscopy after treatment with 100 ng/ml TRAIL for 6 hours. **D.** Detection of TRAIL-induced cleavage of caspase-8 (cas-8), caspase-3 (cas-3), and DFF45 by Western blot on lysates of cells treated with TRAIL (100 ng/mL) for the times indicated. The antibodies are indicated to the *left* and the proteins detected are indicated to the *right*. **E.** Detection of TRAIL-induced mitochondrial release of apoptotic factors by cell fractionation and Western blot. Cells were treated with 100 ng/mL TRAIL for the times indicated and subjected to subcellular fractionation. Cytosolic fractions free of mitochondria were examined for the presence of cytochrome *c* (cyt. *c*), Smac, AIF, XIAP and caspase-9 (cas-9) and β-actin as protein loading control.

denced apoptosis marked by cell surface blebbing (Figure 1C). These results suggest that TRAIL induces cell death mainly through cell surface DR5 receptor expression.

Next, we examined these sensitive cell lines to determine if TRAIL killing occurred through the caspase-8-initiated extrinsic caspase pathway. Caspase-8 cleavage in the DISC occurs in 2 consecutive steps: the first-step cleavage generates large p43 and p41 and small p12 subunits from p55 and p53 precursors and the second step produces a prodomain and active p18 and p10 subunits (35). The active caspase-8 subunits are released from the DISC into the cytosol where they cleave downstream caspase-3 p32 precursor into p20, p17 and p10 subunits (49). Activated caspase-3 in turn proteolytically cleaves its substrates such as DFF45 (18, 33), to execute programmed cell death. To examine this caspase-8-initiated cascade, we treated these cell lines with 100 ng/ml TRAIL and examined the cell lysates looking for caspase cleavage products on Western blots. Both U343MG and LN71 cell lines express endogenous caspase-8 p55 and p53 precursors, which were cleaved into caspase-8 active p18 subunits after exposure to TRAIL for 0.5 to 4 hours (Figure 1D). TRAIL-induced cleavage of caspase-3 from its p32 precursor into p20 and p17 subunits was also demonstrated by Western blotting (Figure 1D). Both the long form (p45) and the short form (p35) of DFF45 precursors were endogenously expressed in the

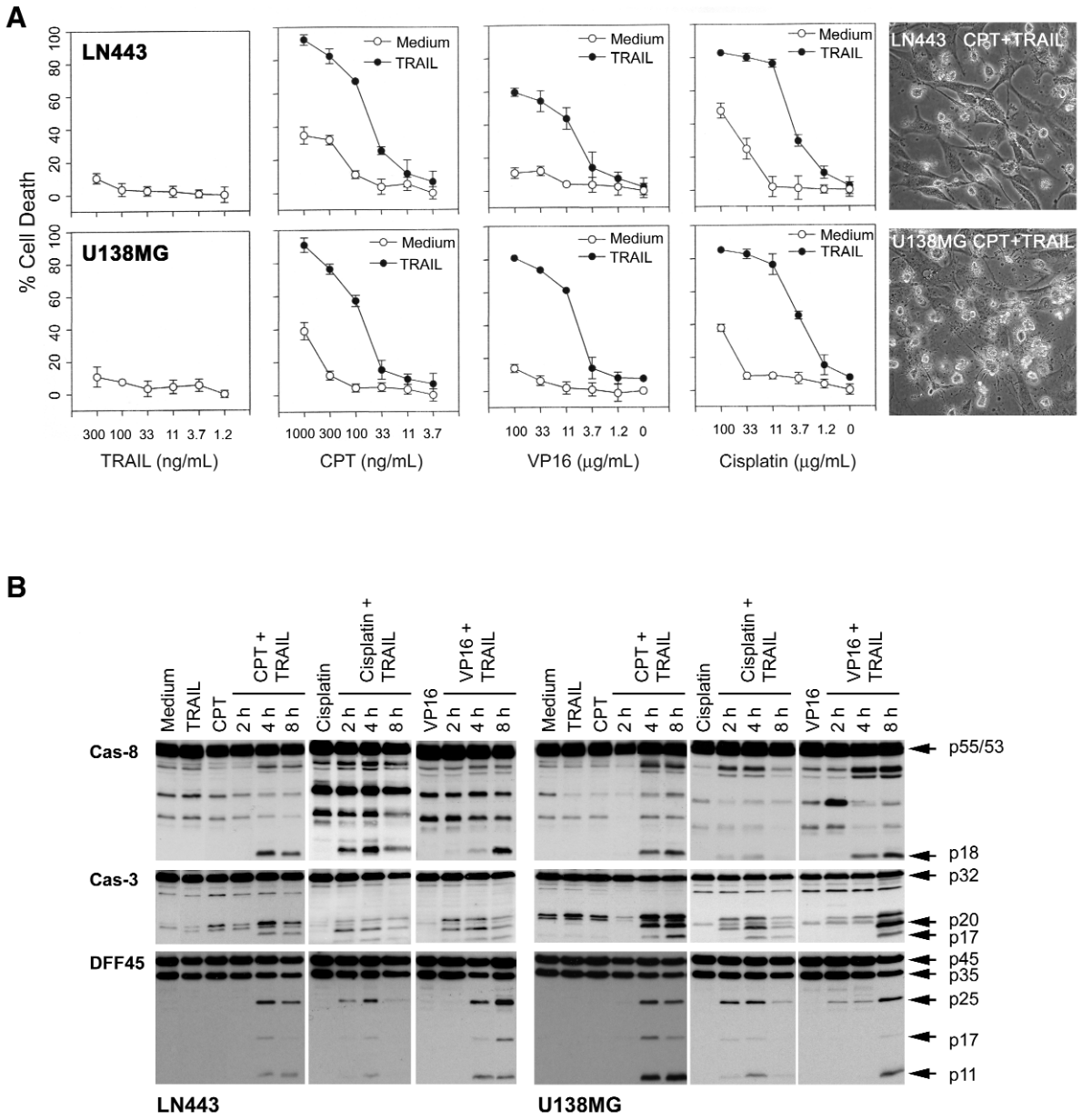


Figure 2. CPT, VP16 and cisplatin sensitize resistant glioma cells to TRAIL-induced caspase-8-initiated caspase cascade. **A.** Effect of the drugs on TRAIL-induced cell death. TRAIL-resistant glioma cell lines (LN443 and U138MG) grown in 96-well plates (3×10^4 cells/well) were treated with CPT, VP16 or cisplatin for 16 hours at the concentrations indicated. Some of the cells were further treated with 100 ng/mL TRAIL for 16 hours. Cell death was determined by a crystal violet assay. Data represents the mean ± SEM (n = 8). These cells were also examined under phase contrast microscopy for cellular apoptosis (the right panel). **B.** Effects of the drugs on TRAIL-induced cleavage of caspase-8, caspase-3 and DFF45. LN443 and U138 cells were treated with TRAIL (100 ng/ml), CPT (100 ng/ml), cisplatin (10 μg/ml) or VP16 (10 μg/ml) for 16 hours, respectively. Some of the cells treated with CPT, cisplatin or VP16 were further treated with 100 ng/ml TRAIL for the times indicated. Cell lysates were subjected to Western blot analysis using antibody to caspase-8 (Cas-8), caspase-3 (Cas-3) and DFF45, as indicated to the *left*. The precursors and cleavage products of the caspases and DFF45 are indicated to the *right*.

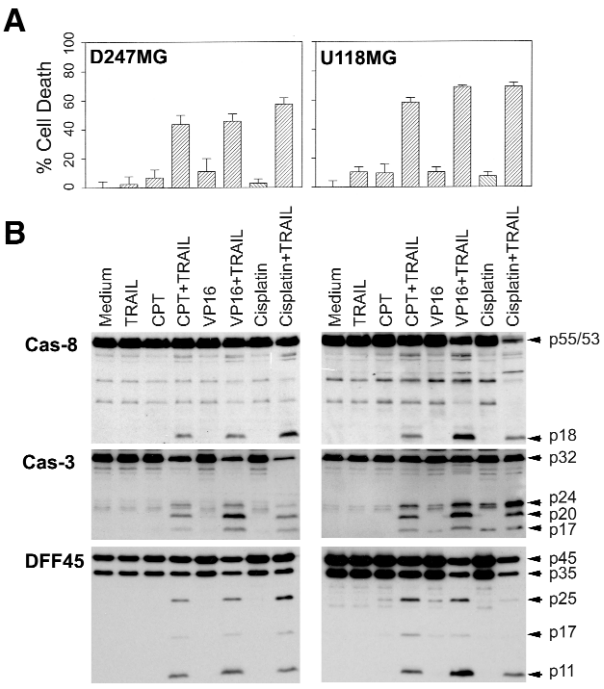


Figure 3. CPT, VP16 and cisplatin sensitize D247MG and U118MG resistant glioma cells to TRAIL. **A.** Effects on TRAIL-induced cell death. Cells grown in 96-well plates (3×10^4 cells/well) were treated with TRAIL (100 ng/ml), CPT (100 ng/ml), cisplatin (10 μg/ml) and VP16 (10 μg/ml) for 16 hours and then incubated with or without TRAIL (100 ng/ml) for additional 16 hours. Cell death was determined by crystal violet assays. Data represents the mean ± SEM (n = 8). **B.** Effects on TRAIL-induced cleavage of caspases and DFF45. Cells were treated with TRAIL (100 ng/ml), CPT (100 ng/ml), cisplatin (10 μg/ml) and VP16 (10 μg/ml) for 16 hours. Some cells treated with CPT, cisplatin and VP16 were further treated with TRAIL (100 ng/mL) for 6 hours. Cell lysates were examined on Western blots using antibodies to caspase-8 (Cas-8), caspase-3 (Cas-3) and DFF45, as indicated to the *left*. The proteins detected are indicated to the *right*.

glioma cells and cleaved into their respective subunits after TRAIL stimulation in the same time frame (Figure 1D).

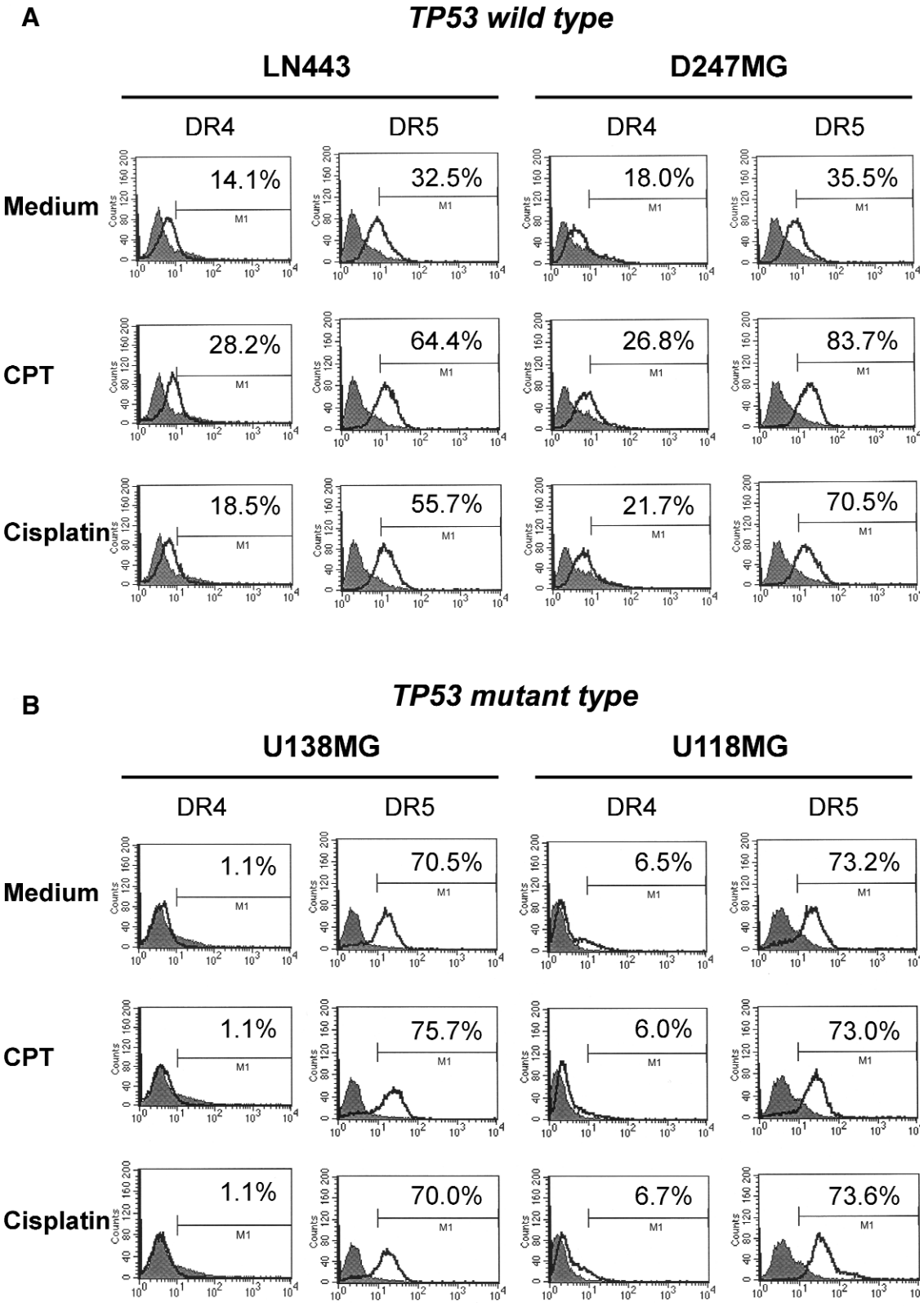
Furthermore, we examined the sensitive glioma cell lines to determine whether TRAIL also triggered activation of the intrinsic mitochondrial pathway. The cell lines were treated with 100 ng/ml of TRAIL and then subjected to subcellular fractionation. The cytosolic fractions free of mitochondria were examined to determine if TRAIL induced mitochondrial release of apoptotic factors into the cytosol. Western blots showed a significant decrease of Bid p22 precursor proteins in both cell lines after exposure to TRAIL (Figure 1E), suggesting that TRAIL induces proteolytic cleavage of Bid. Western

Figure 4. (Opposing page) Flow cytometry analysis of the effects of CPT and cisplatin on DR4 and DR5 expression in TRAIL-resistant *TP53*-wild-type LN443 and D247MG (**A**) and *TP53*-mutated U138MG and U118MG glioma cell lines (**B**). Histograms represent 10^4 gated cells and number indicates percent of positive cells. Shadows represent results from isotype control antibodies. Cell lines were treated or untreated with CPT (100 ng/ml) and cisplatin (10 μg/ml) for 16 hours and then analyzed by flow cytometry for cell surface DR4 and DR5 expression.

blots further revealed mitochondrial release of cytochrome *c*, Smac/DIABLO, and AIF into the cytosol (Figure 1E). TRAIL also induced XIAP cleavage from its p57 precursors into p25 subunits in the cytosol (Figure 1E). In addition, caspase-9 was cleaved from p47 precursors into p37 and p35 subunits in the cytosol (Figure 1E). These results indicate that TRAIL-induced apoptosis also occurs through the intrinsic mitochondrial pathway in sensitive glioma cells.

Cisplatin and CPT sensitize resistant glioma cells to TRAIL-induced apoptosis via the caspase-8-initiated caspase cascade. Many glioma cell lines express TRAIL receptors yet are resistant to TRAIL (19). To overcome the resistance, we screened conventional chemotherapeutic drugs and showed that cisplatin, CPT, and VP16 sensitize resistant glioma cells to TRAIL-induced apoptosis. TRAIL-resistant cell lines LN443 and U138MG were treated for 16 hours with various doses of cisplatin, CPT and VP16. Crystal violet analysis showed only a limited cytotoxic effect of these drugs on the cell lines (Figure 2A). In contrast, further treatment of these cell lines with 100 ng/ml TRAIL for an additional 16 hours resulted in significantly increased cell death, as shown by crystal violet and morphological analysis (Figure 2A). We repeated the experiments on 2 additional resistant cell lines (D247MG, U118MG) with similar results (Figure 3A).

We then examined resistant glioma cells to determine whether drug-induced sensitivity of the cells to TRAIL occurred through the caspase-8-initiated caspase cascade. TRAIL-resistant glioma cell lines LN443, U138MG, D247MG and U118MG were first treated with low doses of CPT (100 ng/ml), cisplatin (10 μg/ml) or VP16 (10 μg/ml) for 16 hours and then stimulated with TRAIL (100 ng/ml). The cell lysates were examined by Western blot for cleavage of caspase-8, caspase-3 and DFF45. Indeed, cleavage products of caspase-8, caspase-3 and DFF45 were detected in the resistant cells treated with the combination of TRAIL and each of the drugs, but not seen in the individual treatments


A
TP53 wild type
LN443
D247MG
DR4
DR5
DR4
DR5
Medium
14.1%
32.5%
18.0%
35.5%
CPT
28.2%
64.4%
26.8%
83.7%
Cisplatin
18.5%
55.7%
21.7%
70.5%
B
TP53 mutant type
U138MG
U118MG
DR4
DR5
DR4
DR5
Medium
1.1%
70.5%
6.5%
73.2%
CPT
1.1%
75.7%
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73.0%
Cisplatin
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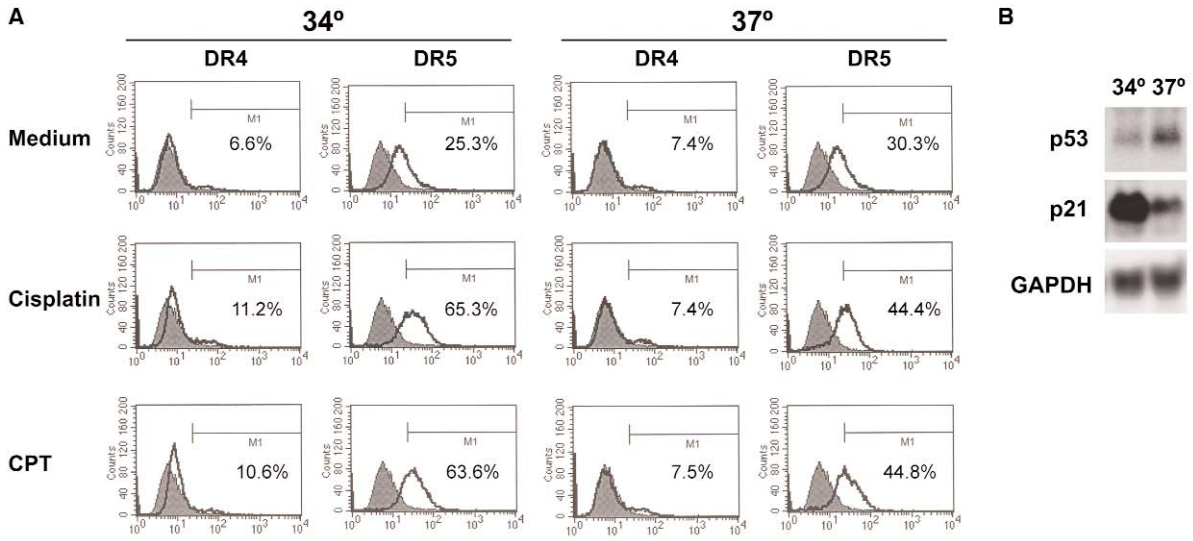

Figure 5. Effects of CPT and cisplatin on DR4 and DR5 expression in temperature-sensitive p53 inducible LN382 cell line. **A.** Flow cytometry analysis. The cells were grown either at 34°C or 37°C for 48 hours and then treated with CPT (100 ng/ml) or cisplatin (10 μg/ml) for 16 hours. **B.** Northern blot analysis of p53 and p21 mRNA expression in LN382 cells. Increased p21 cell cycle inhibitor mRNA level detected in cells incubated at 34°C but not 37°C shows that p53 is transcriptionally active at 34°C.

(Figure 2B; Figure 3B). The results clearly indicate that chemotherapeutic drugs, cisplatin, CPT and VP16 sensitize resistant glioma cells to TRAIL-induced apoptosis through the caspase-8 initiated extrinsic pathway.

CPT and cisplatin down-regulate c-FLIP$_S$ in resistant glioma cells. Cisplatin and VP16 have been reported to upregulate DR5 mRNA expression, thus enhancing TRAIL-induced apoptosis through p53-dependent mechanisms (1, 38, 54). To test this hypothesis in glioma cells, we compared the cell surface DR5 expression of p53-wild type and p53-mutated TRAIL-resistant glioma cell lines (23). These cell lines were treated with low doses of CPT (100 ng/ml) and cisplatin (10 μg/ml). Flow cytometry analysis showed that CPT and cisplatin significantly up-regulated DR5 in p53-wild type LN443 and D247MG cell lines (Figure 4A), but not in p53-mutated U138MG and U118MG cell lines (Figure 4B). Cell surface DR4 expression was slightly up-regulated in p53-wild type, but not in p53-mutated cell lines.

To further confirm that this finding was due to p53, we examined DR4 and DR5 expression in LN382, a glioma cell line containing an endogenous temperature-sensitive p53 mutant. LN382 expresses *TP53* alleles mutated at codon 197 GTG (Val) → CTG (Leu) and the p53 protein in the cells acts as an inactive mutant at 37°C, but as a functional wild-type p53 at 34°C (21). The cell line was incubated either at 37°C or 34°C for 48 hours and treated with either CPT (100 ng/ml) or cisplatin (10 μg/ml) for 16 hours, respectively. These treatments up-regulated slightly DR4, but significantly DR5 cell surface expression on the LN382 cells incubated at 34°C, but not at 37°C (Figure 5A). Induction of p53-wild type activity at 34°C was verified by transcriptional induction of the p21 cell cycle inhibitor mRNA (Figure 5B). The results indicate that CPT and cisplatin up-regulate DR4 and DR5 expression through p53-dependent pathways in glioma cells. However, CPT and cisplatin sensitized both p53-wild type and p53-mutated cell lines to TRAIL (Figures 2, 3), suggesting that these drugs may target different molecules to modulate TRAIL-induced apoptosis in glioma cells.

The fact that CPT and cisplatin sensitize resistant glioma cells to TRAIL-induced apoptosis through the caspase-8-initiated caspase cascade (Figures 2, 3) suggests that the drugs may modulate proteins in the TRAIL-induced DISC. To test this hypothesis, we first examined TRAIL-resistant cell lines for their expression of FADD and caspase-8, as compared with sensitive cell lines (U343MG and LN71), and then whether this expression was altered with the chemotherapeutic drugs. Western blots revealed that FADD and caspase-8 were expressed at consistent levels in TRAIL-sensitive and resistant cell lines and that they remained unchanged after exposure to CPT and cisplatin (Figure 6A). Subsequently, we examined whether the drugs

downregulated c-FLIP, the caspase-8 inhibitor (71) in the resistant cells and thus sensitized them to TRAIL killing. The cells were treated with CPT (100 ng/ml) and cisplatin (10 μg/ml) and Western blot analysis showed down-regulation of c-FLIP$_S$, but not c-FLIP$_L$ (Figure 6B, C). These results suggest that CPT and cisplatin downregulate c-FLIP$_S$ expression, thus sensitizing the resistant cells to TRAIL-induced apoptosis through caspase-8-initiated caspase cascade (Figures 2, 3).

CPT and cisplatin enhance TRAIL-induced activation of the intrinsic pathway through upregulation of Bak. Cisplatin was reported to increase mitochondrial release of cytochrome *c* to enhance TRAIL-induced apoptosis in cancer cells (27). To test this hypothesis in glioma cells, we first examined mitochondrial fractions for cytochrome *c* expression levels in the resistant glioma cell line LN443 after treatment with CPT (100 ng/ml) for 16 hours followed by an additional 2 to 8 hours treatment with TRAIL (100 ng/ml). Western blot analysis showed a significant decrease of cytochrome *c* within the mitochondria in a time-related fashion with the combination treatment (Figure 7A). Examination of the cytosolic fraction showed that the combined CPT/TRAIL treatment triggered release of cytochrome *c*, Smac/DIABLO, and AIF from the mitochondria into the cytosol. Consequently, caspase-9 cleavage was observed (Figure 7A).

To further support these findings, we examined 3 additional TRAIL-resistant glioma cell lines (D247MG, U138MG, and U118MG). The cells were pre-treated with CPT (100 ng/ml) for 16 hours and then treated with TRAIL (100 ng/ml) for an additional 8 hours. Western blot analysis showed decreased cytochrome *c* concentrations in mitochondrial fractions, release of cytochrome *c*, Smac/DIABLO and AIF proteins from the mitochondria into the cytosol, and cleavage of caspase-9 (Figure 7B). The results indicate that CPT sensitizes resistant glioma cells to TRAIL-induced apoptosis through activation of the intrinsic mitochondrial pathway.

The Bcl-2 family members, Bcl-2, Bcl-X, Bax and Bak, regulate mitochondrial membrane potential and release of apoptotic factors (8). To examine this hypothesis in glioma cells, we first compared expression of these proteins in TRAIL-sensitive (U343MG, LN71) and resistant (LN443, D247MG, U138MG, U118MG) cells (Figure 8). Bcl-2 and Bax were expressed at consistent levels in all cell lines and treatment with VP16, cisplatin or CPT did not alter expression of these proteins in the resistant cell lines. Bcl-X was expressed in the long

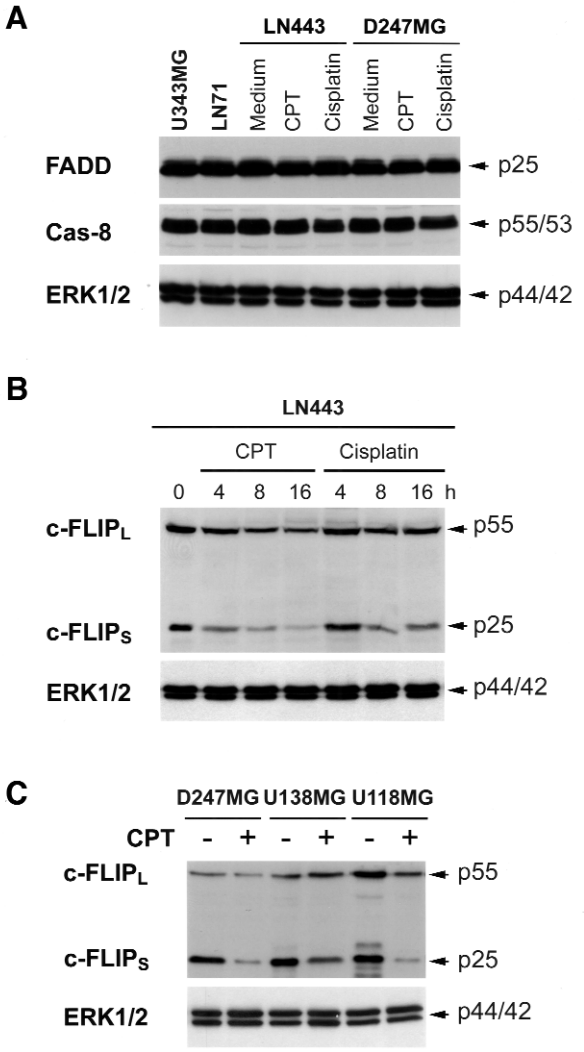

Figure 6. Western blot analysis of the effects of CPT and cisplatin on intracellular FADD, caspase-8 and c-FLIP expression in TRAIL-resistant glioma cell lines. All the cells were treated or untreated with CPT (100 ng/ml) or cisplatin (10 μg/ml) for 16 hours or the times indicated. **A.** FADD and caspase-8 expression. **B, C.** c-FLIP$_L$ and c-FLIP$_S$ expression. The antibodies used are indicated to the *left* and the proteins detected in the *right*. ERK1/2 was used as a loading control.

form (Bcl-X$_L$, p26) and the short form (Bcl-X$_S$, p17) in all cell lines except LN443 that lacked Bcl-X$_S$. Expression of Bcl-X isoforms was largely unaffected by drug treatment except for a slight decrease in Bcl-X$_L$ with CPT treatment in LN443 and U138MG. Bak was expressed in all cell lines, but upregulated in the resistant cell lines after treatment with CPT, cisplatin and VP16. These results suggest that Bak contributes in

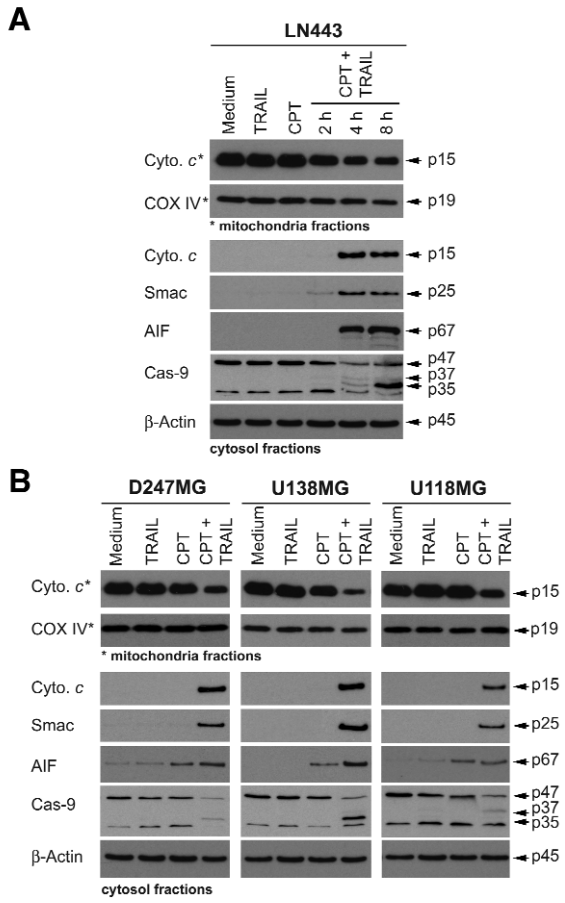

Figure 7. CPT effects on TRAIL-induced activation of mitochondrial pathways in glioma cells. **A.** LN443 cells were pre-treated with CPT (100 ng/ml) for 16 hours followed or not by 2 to 8 hours treatment with TRAIL (100 ng/ml). The mitochondrial fractions were examined on Western blots for cytochrome *c* (Cyt. *c*) using COX IV as protein loading control. The cytosolic fractions were examined for cytochrome *c*, Smac, AIF, cleavage of caspase-9 (Cas-9). β-Actin was used as loading control. **B.** D247MG, U138MG, and U118MG cells were pre-treated with CPT (100 ng/ml) for 16 hours with or without further treatment with TRAIL (100 ng/ml) for 8 hours. The mitochondrial and cytosolic fractions were examined as in A above.

activating the TRAIL-induced mitochondrial pathway in the resistant glioma cell lines.

CPT and cisplatin enhance TRAIL-induced apoptosis in primary glioma cultures through both extrinsic and intrinsic pathways. To further explore the translation ability of these findings to patients with malignant gliomas, we examined whether TRAIL, alone or in combination with drugs such as CPT, could kill freshly explanted human glioma cells. Three primary

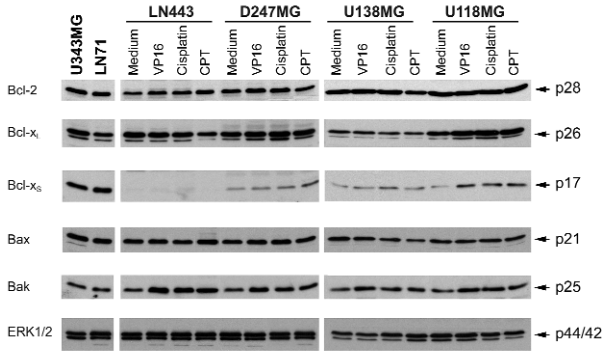

Figure 8. Effects of CPT, cisplatin, VP16 on Bcl-2 family protein expression. TRAIL-resistant LN443, D247MG, U138MG, and U118MG glioma cell lines were treated with CPT (100 ng/ml), cisplatin (10 g/ml) and VP16 (10 g/ml) for 16 hours and, together with untreated cells, subjected to Western blot analysis. TRAIL-sensitive U343MG and LN71 glioma cell lines were included in the analysis. Antibodies are indicated to the *left* and the proteins detected indicated to the *right*. ERK1/2 was used as protein loading control.

cultures (ED326BT, ED273BT, and ED189BT) prepared from explants removed from patients with glioblastomas were treated at passage 3 to 5 with CPT (100 ng/ml) or TRAIL (100 ng/ml) for 16 hours. ED326BT was sensitive, whereas the other two were resistant to TRAIL-induced cell death, as determined by crystal violet assay (Figure 9A). However, resistance to TRAIL was overcome by pretreatment of the resistant cultures with CPT (100 ng/ml) for 16 hours (Figure 9A). Next, we examined activation of the caspase-8-initiated caspase cascade in these cells (Figure 9B). Western blots detected the cleavage products of caspase-8, caspase-3, caspase-9 and DFF45 in the cells that were exposed to TRAIL and CPT. These results indicate that TRAIL alone or in combination with CPT is sufficient to trigger apoptosis in primary human glioma cultures through both extrinsic and intrinsic apoptotic pathways.

Discussion

Malignant gliomas of astrocytic origin, including astrocytoma, anaplastic astrocytoma and glioblastoma, are the most common human primary brain tumors. Despite intensive research, current treatment modalities have not significantly improved patient survival over the last three decades. Recently, we have shown that TRAIL kills malignant glioma cells through induction of apoptosis, providing a new therapeutic strategy for these tumors (19, 71). Due to the heterogeneity of the tumors, many glioma cells are resistant to TRAIL-induced apoptosis, even though they express appropri-

ate components of the death machinery (19, 71). Here, we demonstrate that treatment of resistant glioma cells with conventional chemotherapeutic drugs such as cisplatin and CPT results in the release of molecular inhibition of death receptor-mediated extrinsic and mitochondria-involved intrinsic pathways, thus sensitizing the resistant glioma cells to TRAIL-induced apoptosis.

Of the two TRAIL death receptors, DR4 and DR5, malignant glioma cells mainly express DR5 and they undergo apoptosis after exposure to TRAIL alone or in combination with chemotherapeutic drugs (1, 19, 40). Chemotherapeutic drugs such as cisplatin, doxorubicin and VP16 have been reported to upregulate DR5 transcripts and thus cause synergistic cytotoxicity with TRAIL in glioma (12, 40) and sarcoma (11). Other studies by flow cytometry, however, showed constitutive DR5 protein expression on the cell surfaces of colon cancer (27) and melanoma (57) irrespective of drug treatment. DR5 was initially cloned as a DNA-damage-inducible and p53-regulated molecule (55), but is also a glucocorticoids and interferon-γ inducible gene in a p53-independent fashion (36). We found that cisplatin and CPT up-regulate cell surface expression of DR5 in p53-wild-type, but not in p53-mutated resistant glioma cell lines. Regardless of their effect on DR5 expression, these chemotherapeutic drugs sensitized resistant glioma cells to TRAIL-induced apoptosis, indicating the existence of alternative molecular mechanisms for the modulation of TRAIL resistance in glioma cells.

We have reported previously that TRAIL interacts with DR5 to recruit FADD and caspase-8 to assemble a DISC, in which caspase-8 is activated and thereby initiates apoptosis in sensitive glioma cells (71). We further showed that in TRAIL resistant glioma cells c-FLIP$_L$ and c-FLIP$_S$ are highly expressed and are recruited to the DISC where they inhibit caspase-8 cleavage, thus preventing caspase-8-initiated apoptosis (71). Studies of c-FLIP proteins in cancer cells have produced controversial results mainly due to the usage of anti-FLIP rabbit serum that only recognizes c-FLIP$_L$ (19, 22, 73). Our studies used anti-c-FLIP monoclonal antibody (NF6 clone) that recognizes both c-FLIP$_L$ and c-FLIP$_S$ (50). We now show that treatment of resistant glioma cells with appropriate chemotherapeutic drugs restores TRAIL-induced caspase-8 cleavage and downstream activation of the caspase cascade, thus sensitizing the cells to TRAIL-induced and caspase-8-initiated apoptosis.

Recently, Smac/DIABLO agonists were reported to sensitize TRAIL-induced apoptosis in glioma cells (13). This may occur through Smac/DIABLO interaction

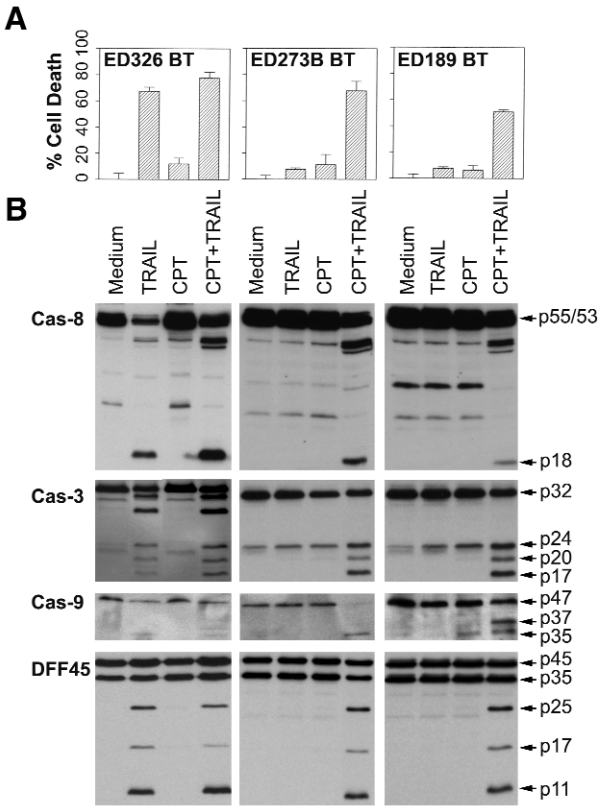

Figure 9. Synergistic effects of CPT on TRAIL-induced apoptosis in primary glioma cultures. **A.** TRAIL-induced cell death. Cells in early (3-5) passages were grown in 96-well plates (3×10^4 cells/well) overnight and treated with CPT (100 ng/ml) for 16 hours. Some of the cells were further treated with TRAIL (100 ng/ml) for 16 hours. Cell death was determined by crystal violet assays and the data represents the mean ± SEM (n = 6). **B.** TRAIL-induced cleavage of caspases and DFF45. The primary cultures were treated with CPT (100 ng/ml) for 16 hours. Some of the cells were further treated with TRAIL (100 ng/ml) for 6 hours. The cells were then analyzed on Western blots. The antibodies are indicated to the *left* and the proteins detected indicated to the *right*.

with XIAP, which is highly expressed in glioma cells (64). These results suggest that TRAIL may induce activation of intrinsic mitochondrial pathways, which may amplify and rescue TRAIL receptor-mediated caspase-8-initiated activation of caspase cascade in the extrinsic pathway. Indeed, we show that TRAIL triggers Bid cleavage and mitochondrial release of cytochrome *c*, AIF and Smac/DIABLO, resulting in XIAP cleavage and activation of caspase-9 and caspase-3 in TRAIL-sensitive glioma cells. The chemotherapeutic drugs such as CPT and cisplatin downregulate c-FLIP to release its inhibition on caspase-8 cleavage in the DISC and thus sen-

sitize resistant glioma cells to TRAIL-induced activation of the mitochondrial pathway.

The Bcl-2 family members, Bcl-2, Bcl-X, Bax and Bak, have been reported to modulate mitochondrial membrane potential and release of apoptotic factors that control TRAIL-induced apoptosis (20, 37, 41, 47, 60). Here we show that Bcl-2, Bcl-X, Bax and Bak are expressed in both sensitive and resistant glioma cells and their expression levels do not correlate to the sensitivity of the cell lines to TRAIL-induced apoptosis. Pretreatment of the resistant cell lines with chemotherapeutic drugs upregulated Bak, but had no effects on Bax, Bcl-2, and Bcl-X expression. These results suggest that chemotherapeutic drugs may modulate mitochondrial membrane potential through Bak and thereby facilitate TRAIL-induced apoptosis. The molecular mechanisms by which Bak interacts with other Bcl-2 proteins and may modulate mitochondrial release of apoptotic factors remain to be investigated in glioma cells.

Several lines of evidence suggest that TRAIL may be a safe and effective therapeutic agent for malignant gliomas as this agent appears to be non-toxic to normal human astrocytic cells (19). Local delivery of soluble TRAIL (48), TRAIL-expressing adenoviral vector (30) and TRAIL-secreting neural stem cells (10) have all been shown to induce apoptosis in intracranial gliomas. Systemic administration of soluble TRAIL along with chemotherapeutic drugs inhibits glioma growth without causing neurological impairment or damage to other normal tissues in mice (40). Here we further show that TRAIL alone or in combination with specific chemotherapeutic drugs can induce apoptosis in primary cultures of human glioblastomas, thus providing further support for the use of this novel therapeutic approach in patients with malignant gliomas.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research (MOP49621, to C.H.) and the Alberta Heritage Foundation for Medical Research (AHFMR to C.H.) and the USA National Institutes of Health (CA86335; CA87830; and NS41403 to E.G.V.M.). Dr Chunhai Hao is a recipient of the AHFMR Clinical Investigator Award.

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