

# Direct Stimulation of Apoptotic Signaling by Soluble Apo2L/Tumor Necrosis Factor-related Apoptosis-inducing Ligand Leads to Selective Killing of Glioma Cells<sup>1</sup>

Ian F. Pollack,<sup>2</sup> Melanie Erff, and Avi Ashkenazi

Department of Neurosurgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213 [I. F. P., M. E.], and Department of Molecular Oncology, Genentech, Incorporated, South San Francisco, California 94080 [A. A.]

## ABSTRACT

Apo2 ligand tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) is a member of the tumor necrosis factor family that interacts with cell surface “death receptors” (DR4 and DR5) to initiate programmed cell death. Apo2L/TRAIL also binds to “decoy” receptors (DcR1 and DcR2) that can antagonize its interaction with DR4 and DR5. In recent studies, Apo2L/TRAIL has been noted to produce selective toxicity toward certain neoplastic cells *versus* normal cells. The decoy receptors may in part contribute to this selectivity, because they are expressed in various normal tissues but are present at low or undetectable levels in certain types of neoplastic cells. In the current study, we examined the potential therapeutic applicability of recombinant soluble Apo2L/TRAIL by investigating its effects *in vitro* and *in vivo* against a series of cell lines derived from malignant gliomas, which are often resistant to conventional treatment modalities. In cell proliferation assays, Apo2L/TRAIL produced a striking decrease in cell numbers, with a median inhibitory concentration of 30–100 ng/ml, in the *TP53* wild-type high-grade glioma cell lines U87 and A172, the *TP53*-mutated T98G, and the *TP53*-deleted LN-Z308. In contrast, no significant effects were observed in non-neoplastic astrocytes at concentrations up to 3000 ng/ml. Clonogenic assays showed that exposure to Apo2L produced a time-dependent decrease in the viability of glioma-derived cell lines. This correlated with the induction of apoptosis as assessed by a terminal transferase-catalyzed *in situ* end-labeling assay. Pretreatment of the cells with the caspase inhibitors Acetyl-Asp-Glu-Val-L-aspartic acid aldehyde or Acetyl-Tyr-Val-Ala-Asp-chlormethylketone (200  $\mu$ M) largely eliminated the effects of Apo2L/

TRAIL. Administration of Apo2L/TRAIL (0.3, 1, 3, 10, and 30 mg/kg/day for 7 days via i.p. infusion) to nude mice harboring established intracranial U87 xenografts produced a significant, dose-dependent prolongation of survival *versus* control animals. Survival in the control group was  $27 \pm 1.7$  days, compared with more than 50 days in each of the treatment groups ( $P < 0.001$ ). At the 30 mg/kg dose level, 100% of animals survived for 120 days without evidence of tumor, a substantial improvement in comparison with lower dose levels ( $P < 0.01$ ). No overt toxicity was apparent even at the highest Apo2L dose. We conclude that soluble Apo2L/TRAIL is effective in inducing apoptosis in high-grade glioma cells *in vitro*. Because this ligand appears to exhibit selective cytotoxicity for glioma cells *versus* non-neoplastic cells *in vitro* and demonstrates significant activity *in vivo* when administered systemically in an otherwise uniformly fatal central nervous system glioma model system, Apo2L may constitute a useful therapeutic agent for these challenging tumors.

## INTRODUCTION

Neoplasia results in part from suppression of the normal intracellular mechanisms for triggering apoptotic signaling (1, 2). In malignant glioma, the most common form of primary central nervous system neoplasia (3), a variety of factors have been implicated in mediating resistance to apoptosis. In particular, the p53 protein, which is a critical facilitator of DNA damage-induced apoptosis (2, 4, 5), is mutated or deleted in as many as 50% of high-grade gliomas (6, 7). In addition, glioma cells are able to avoid the induction of apoptosis resulting from mitogen depletion as a result of their autocrine growth stimulation through a number of growth factor/receptor interactions, including platelet-derived growth factor (8, 9), fibroblast growth factor (10, 11), and insulin-like growth factor (12, 13), as well as their frequent overexpression of a mutated, constitutively activated epidermal growth factor receptor (14).

The suppression of endogenous apoptosis-triggering mechanisms allows glioma cells with severe DNA damage to survive and propagate, transmitting progressively more malignant features to their progeny. This may in part account for the resistance of gliomas to conventional chemotherapy and radiotherapy (3, 15). However, because the effector arm of the apoptotic machinery often remains intact, these cells can be induced to undergo apoptosis by direct stimulation of cell death receptors of the TNFR<sup>3</sup> superfamily (16, 17), such as Fas (Apo1) and

Received 2/18/00; revised 12/13/00; accepted 2/6/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported in part by NIH Grants NS01810 and NS37704 and a Grant from the Copeland Fund of the Pittsburgh Foundation (to I. F. P.).

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Neurosurgery, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213. Phone: (412) 692-5881; Fax: (412) 692-5921; E-mail: Pollaci@chplink.chp.edu.

<sup>3</sup> The abbreviations used are: TNFR, tumor necrosis factor receptor; Apo2L, Apo2 ligand; TRAIL, TNF-related apoptosis-inducing ligand; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

TNFR1 (18). Unfortunately, the clinical applicability of this approach has been limited. Attempts to kill neoplastic cells *in vivo* by stimulating TNFR1 or Fas/Apo1 have produced excessive toxicity. TNF causes a severe systemic inflammatory syndrome, whereas Fas activation causes lethal liver damage (16, 19, 20).

More recent studies (21–23) have identified a TNF-related molecule, referred to as Apo2L or TRAIL, that interacts with two TNFR-related cell death receptors (DR4 and DR5) and appears to selectively mediate apoptotic signaling on certain neoplastic cells relative to normal cells (24–29). One mechanism that may in part account for this selectivity is the preferential expression of “decoy” receptors, such as DcR1 and DcR2, in normal *versus* neoplastic cells (25–27, 29–33). These decoy receptors compete effectively with DR4 and DR5 for extracellular ligand binding but lack a functional cytoplasmic domain and, thus, cannot initiate apoptotic signaling.

The aims of the present study were to examine the utility of recombinant soluble Apo2L/TRAIL for selectively killing glioma cells *versus* non-neoplastic astrocytes *in vitro* and to evaluate the activity of this ligand for inhibiting tumor growth in a central nervous system tumor model system. The rationale for these studies was supported by recent observations that a majority of glioma cell lines express agonist receptors for Apo2L/TRAIL (*i.e.*, DR4 and DR5) but express low or undetectable levels of antagonist receptors (*i.e.*, DcR1 and DcR2; Ref. 34). Given the poor sensitivity of glial tumors to conventional therapeutic approaches (3, 15), the identification of a novel strategy for selectively inducing apoptosis in glioma cells could provide a powerful therapeutic tool for this type of cancer. These experiments demonstrated potent induction of cytotoxicity in glioma cells by Apo2L/TRAIL *in vitro* with no obvious toxicity in non-neoplastic astrocytes, as well as significant antitumor activity *in vivo*, which supports the potential therapeutic utility of this agent.

## MATERIALS AND METHODS

**Cell Culture.** The *TP53*-wild-type human malignant glioma cell lines A172 and U87 and the *TP53*-mutated T98G were obtained from the American Type Culture Collection. LN-Z308, a *TP53*-deleted glioma cell line, was kindly provided by Dr. Nicolas deTribolet (University of Lausanne, Lausanne, Switzerland). Each of these cell lines was maintained in growth medium consisting of  $\alpha$ -MEM supplemented with L-glutamine, ribonucleosides, deoxyribonucleosides, 10% FCS (Life Technologies, Inc.), and the following antimicrobial agents: 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air and subcultured every 4 to 7 days with 0.25% trypsin in HBSS (Life Technologies, Inc.).

A low passage non-neoplastic astrocyte cell line (N49) was derived from a temporal lobectomy specimen in a patient with intractable seizures. A portion of the surgical specimen was

finely dissected to remove blood and leptomeninges, cut into pieces approximately 1 mm<sup>3</sup> in diameter, and dissociated by incubation for 1 h at 37°C in 0.25% trypsin, 0.02% DNase. Dissociated cells were filtered through 100- $\mu$ m nylon mesh and then cultured for 48 h in growth medium. Medium containing nonadherent cells was removed, and fresh medium was applied. Cells were split after reaching confluence. Cultures were found to contain more than 95% glial fibrillary acidic protein positive cells and were stored as aliquots in liquid nitrogen, so that studies could be performed on cells that had been passaged less than 10 times in culture.

**MTS Assay of Cell Numbers After Apo2L/TRAIL Treatment.** A colorimetric assay was used in each of the aforementioned cell lines to assess the effect on cell numbers of a range of concentrations of homotrimeric soluble Apo2L/TRAIL (amino acids 114–281), which was produced in *Escherichia coli* (Genentech, South San Francisco, CA). Details regarding the expression and purification procedures used have been reported previously (35). For these studies,  $5 \times 10^4$  cells were plated, grown for 12 h in 100  $\mu$ l of growth medium in 96-well microtiter plates (Costar, Cambridge, MA), and then treated for 4 days with various concentrations of Apo2L. A stock solution of 0.62 mg/ml in a vehicle of 20  $\mu$ M sodium acetate, 8% trehalose, and 0.01% Tween (pH 5.5) was diluted in growth medium for all of the studies. Control cells were treated with the concentration of vehicle used at the highest Apo2L/TRAIL dose level. All of the studies were performed in triplicate on two separate occasions.

After a 4-day incubation period, the number of viable cells was determined by measuring the bioreduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of the electron-coupling reagent phenazine methosulfate. MTS and phenazine methosulfate were added to the culture wells as per the supplier's protocol (Promega, Madison, Wisconsin), and the mixture was incubated for 3 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Absorbance at 490 nm was measured using an ELISA microplate reader. The assay was linear over the range of cell numbers examined. Cell counts were calculated by direct comparison of the measured absorbances with those obtained in triplicate wells containing a range of predetermined cell numbers, which were subjected to the above assay in parallel with the test samples in each study.

**Clonogenic Assay.** A direct assessment of the effect of selected concentrations of Apo2L/TRAIL on cell viability was performed using a clonogenic assay. For these studies, 250 U87, T98G, and LN-Z308 cells were plated and, after an overnight attachment period, grown as described above with selected concentrations of Apo2L/TRAIL for periods of 6 h and 4 days. Each of these cell lines has a plating efficiency in the range of 35 to 45%. After the Apo2L/TRAIL treatment period, the cells were washed with Apo2L-free medium and grown in serum-supplemented medium for an additional 2-week period. The plates were then stained with crystal violet, and colonies were counted. All of the studies were performed on two separate occasions.

**Terminal Transferase-catalyzed *in Situ* End-labeling Technique.** The percentage of U87 glioma cells and N49 non-neoplastic astrocyte cells undergoing apoptosis after 6, 12, 24, 48, 72, and 96 h of inhibitor treatment was examined using

sulfophenyl)-2H-tetrazolium; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-L-aspartic acid aldehyde; Ac-YVAD-CMK, acetyl-Tyr-Val-Ala-Asp-chloromethylketone.

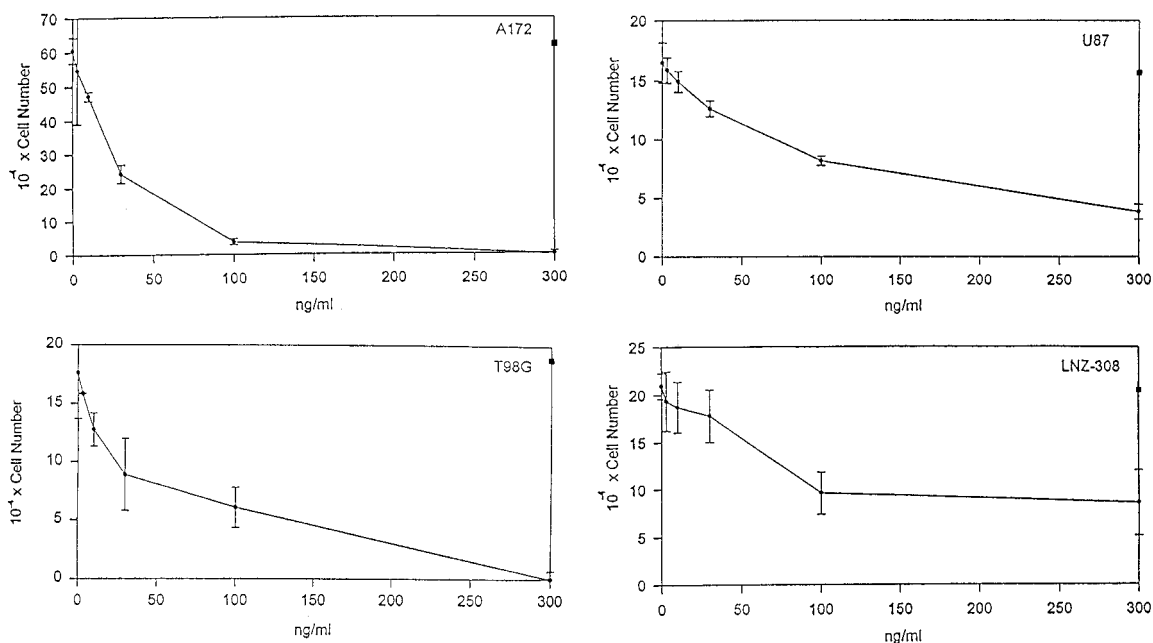


Fig. 1 Effect of Apo2L on growth of glioma cell lines. The relationship between Apo2L concentration and cell numbers, as assessed by a spectrophotometric measurement of MTS bioreduction, is illustrated in the U87, A172, T98G, and LN-Z308 malignant glioma cell lines. Points represent the mean of three measurements; bars,  $\pm$  SD. Y axes vary between cell lines to illustrate the response range observed. No significant inhibition was detected in control cells treated with the concentration of vehicle used at the highest Apo2L/TRAIL dose level; control values are indicated as ■ on the right border of each graph. All of the results were confirmed in a second, independent experiment.

terminal transferase-catalyzed *in situ* end-labeling of endonuclease-cleaved DNA fragments. Control cells were treated with vehicle alone. This technique (ApopTag Plus *In Situ* Apoptosis Detection Kit; Oncor, Gaithersburg, MD) stains apoptotic cells by immunoperoxidase detection of digoxigenin-labeled genomic DNA fragments. Negative and positive controls were included with all of the samples.

Cells ( $6 \times 10^4$ ) were plated on 8-well chamber slides and, after attachment, treated with Apo2L/TRAIL. At the above time points, cells were washed in PBS (pH 7.4), fixed in 4% neutral buffered formalin for 10 min at room temperature, and washed again in PBS. After quenching of endogenous peroxidase, slides were washed in PBS and subjected to terminal deoxynucleotidyl transferase-catalyzed end labeling as per the supplier's protocol. This approach adds digoxigenin-nucleotide residues to both ends of the nucleosome-sized apoptotic DNA fragments. For negative controls, water instead of terminal deoxynucleotidyl transferase enzyme was applied. After the labeling reaction was completed, slides were washed in PBS, and end-labeled fragments were localized using an anti-digoxigenin antibody-peroxidase conjugate. Binding was visualized using diaminobenzidine as a substrate. Specimens were then washed in PBS and, subsequently, in distilled water and counterstained with methyl green. The specimens were then washed, dehydrated in xylene, and mounted. Cells were considered to have apoptotic staining if they exhibited labeling in the context of morphological evidence of condensed nuclear fragments. Direct counting of apoptotic and nonapoptotic cells was performed using an Olympus BH-2 microscope. At least 500 cells were counted/slide.

**The Effect of Caspase Inhibition on Apo2L/TRAIL-mediated Cytotoxicity.** To confirm that the effects of Apo2L/TRAIL on glioma cells were mediated by signaling through caspase cascades, rather than by a nonspecific toxic effect of the compound, we examined the effect of inhibiting caspase 1 (interleukin-1 $\beta$ -converting enzyme) and caspase 3 (apopain; CPP32) on Apo2L-induced cytotoxicity. For these studies, cells were preincubated for 30 min with inhibitors of caspase 1 (Ac-YVAD-CMK) or caspase 3 (Ac-DEVD-CHO) at concentrations of 200  $\mu$ M each (36). Both inhibitors were obtained from Bachem Bioscience Inc. (King of Prussia, PA). After the preincubation period, cells were treated with various concentrations of Apo2L for 4 days. The caspase inhibitors were added again to the medium after 48 h of incubation. Cell numbers were then determined at the conclusion of the incubation period using an MTS cell quantitation assay, as noted above.

**Assessment of Antiproliferative Activity in a Nude Mouse Glioma Model.** *In vivo* assessment of the effect of Apo2L on glioma growth was performed using a nude mouse intracranial model of the U87 glioma cell line (37). Four-week-old nu/nu mice (obtained from the National Cancer Institute) were anesthetized with ketamine (100 mg/kg) and acepromazine (0.02 mg/kg) and immobilized in a stereotactic frame. Through a small right paramedian scalp incision, an opening was made in the frontal bone using a 20-gauge needle tip. U87 cells ( $2 \times 10^5$ ) in 10  $\mu$ l of PBS were injected stereotactically into the right frontal lobe to a depth of 2.5 mm using a Hamilton syringe. This dose of cells produces virtually 100% tumor take in the nude mouse system in our laboratory, as well as elsewhere (37), and

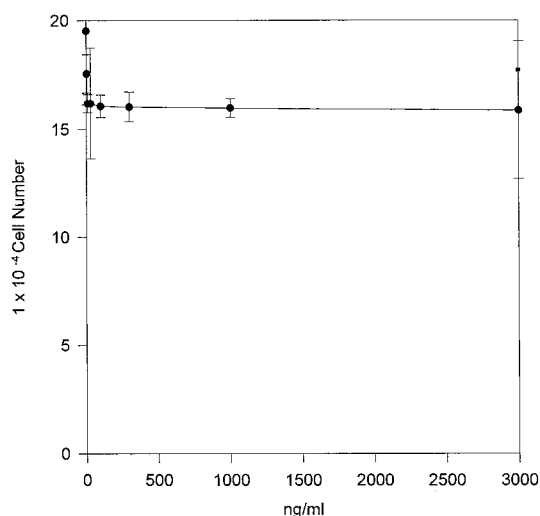


Fig. 2 Effect of Apo2L on growth of a non-neoplastic astrocyte cell line. The relationship between Apo2L concentration and cell numbers is assessed as in Fig. 1. Values in control cells treated with the concentration of vehicle used at the highest Apo2L/TRAIL dose level are indicated on the right border of the graph.

typically induces tumors that manifest with contralateral hemiparesis within 3 to 4 weeks.

Seven days after tumor implantation, animals were randomly chosen to receive either vehicle ( $n = 12$ ) or one of five doses of Apo2L (0.3, 1, 3, or 10 mg/kg/d;  $n = 4$  for each group; or 30 mg/kg/d;  $n = 6$ ), administered daily for 7 days by i.p. bolus infusion in a volume of 350  $\mu$ l.

The mice were examined at least three times/week and were sacrificed when they manifested severe hemiparesis or >20% weight loss. The brains were examined to confirm that the cause of death was tumor growth (rather than infection) in each case. Survival curves for the drug-treated and control animals were compared using a rank-sum test, and survival durations in the treatment and control groups were compared using Student's  $t$  test. All of the animal studies were approved by the Animal Research and Care Committee of the Children's Hospital of Pittsburgh.

## RESULTS

A colorimetric assay was performed to determine the effect of a wide range of Apo2L/TRAIL concentrations on cell numbers after a 4-day culture period in a series of malignant glioma cell lines and non-neoplastic astrocytes. These studies demonstrated a substantial drop-off in viable cell numbers in each of the four glioma cell lines tested with a median effective concentration of 30 to 100 ng/ml in the *TP53* wild-type A172 and U87 cell lines, the *TP53*-mutated T98G cell line, and the *TP53*-deleted LN-Z308 cell line. Representative results for each cell line are shown in Fig. 1. No significant decrease in cell numbers was observed in control cells treated with the concentration of vehicle used at the highest Apo2L/TRAIL dose level. Although these studies showed some variation in the degree to which cell numbers were decreased by Apo2L/TRAIL treatment, this effect did not correlate with differences between the cell lines in the

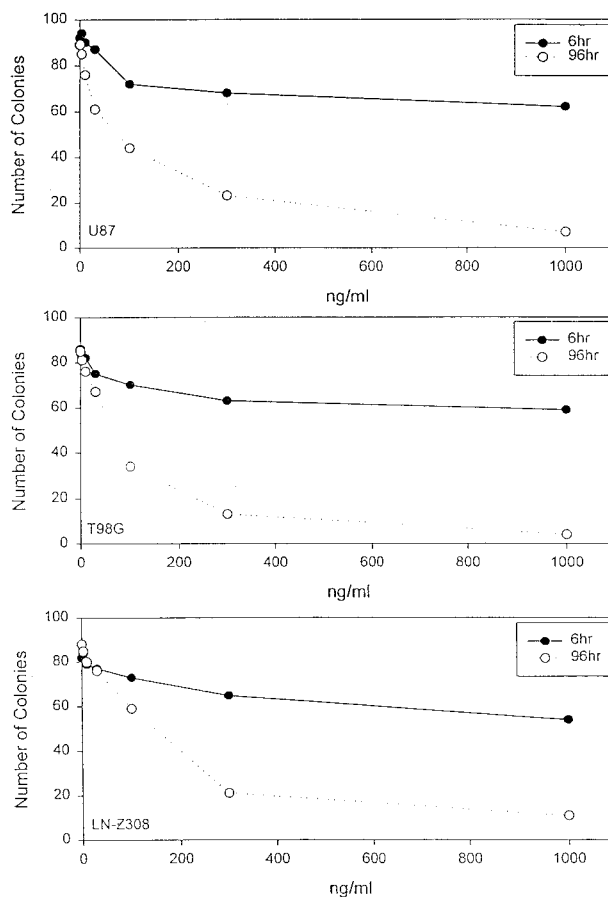


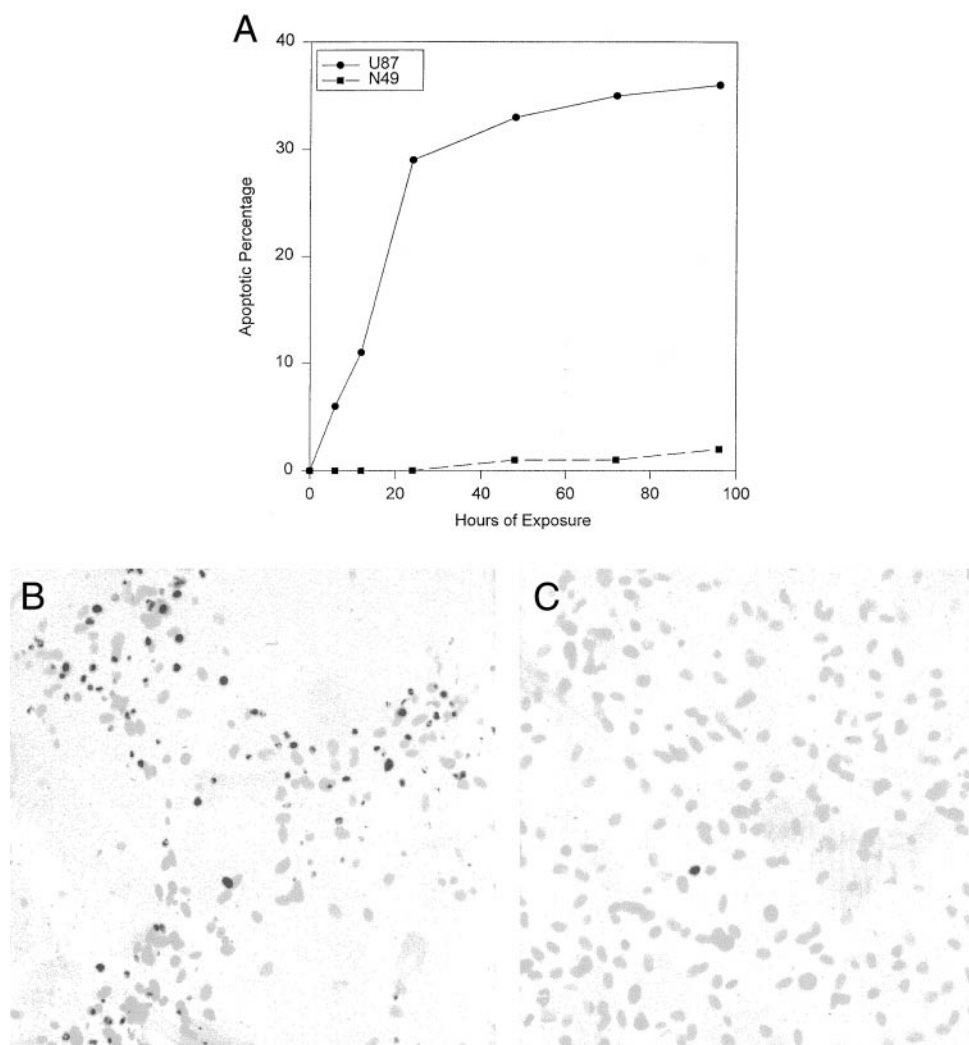
Fig. 3 Effect of Apo2L on clonogenic growth of glioma cell lines. The relationship between colony counts and Apo2L concentration in the U87, T98G, and LN-Z308 cell lines is shown. Cells were treated with Apo2L for 6 and 96 h and then grown for an additional 14 days in the absence of the ligand. Colonies were then counted. These results were confirmed in a second, independent experiment.

patterns of agonist or antagonist receptor expression as assessed by reverse transcription-PCR (data not shown).

In contrast to the striking decline in cell numbers seen in the four glioma cell lines, Apo2L/TRAIL produced no significant effect on cell numbers in a non-neoplastic astrocyte cell line, even with concentrations as high as 3000 ng/ml (Fig. 2).

The inhibitory effects observed in the glioma cell lines were associated with the induction of cytotoxicity by Apo2L/TRAIL, as evidenced by a concentration-dependent and exposure time-dependent decline in clonogenic activity in the *TP53* wild-type U87 cell line, the *TP53* mutant T98G cell line, and the *TP53*-deleted LN-Z308 cell line (Fig. 3). Although there was only a modest decline in clonogenic activity with 6-h exposures, 96-h treatment intervals produced a sharp decrease in clonogenic activity with concentrations in the range of 30 to 300 ng/ml (Fig. 3). These results were confirmed in a second, independent experiment.

This cytotoxicity correlated with the induction of apoptosis as assessed by a terminal transferase-catalyzed *in situ* end-labeling reaction. U87 glioma cells treated with Apo2L/TRAIL



**Fig. 4** Time course of apoptosis induction by Apo2L (A). Apoptotic percentage, assessed by terminal transferase-catalyzed end labeling of DNA fragments, after various intervals of exposure to 300 ng/ml of Apo2L in U87 malignant glioma cells and N49 non-neoplastic astrocyte cells is shown. Whereas the apoptotic labeling percentage reached 30% in U87 glioma cells after 24 h of exposure to Apo2L/TRAIL and remained elevated (96-h result is illustrated in B), the labeling percentage remained less than 3% in non-neoplastic astrocytes, even after extended intervals of Apo2L treatment, as well as in vehicle-treated control glioma cells (96-h result is shown in C).

showed a significant increase in apoptotic labeling that was not apparent in non-neoplastic astrocytes or in vehicle-treated control cells (Fig. 4). The percentage of U87 cells that exhibited apoptotic labeling increased approximately 5-fold from 6 to 29% between 6 and 24 h after beginning Apo2L treatment and continued to increase more slowly thereafter (Fig. 4, A and B), whereas non-neoplastic astrocytes (N49 cells) exhibited apoptotic labeling percentages of less than 3% at each of the time points examined. As illustrated in Fig. 4C, the apoptotic percentage of vehicle-treated control U87 cells was also less than 3%, even at the 96-h time point.

The cytotoxic effects of Apo2L were associated with activation of caspases, which are downstream mediators of apoptotic signaling. Inhibition of these proteases by the tetrapeptide inhibitors Ac-YVAD-CMK and Ac-DEVD-CHO substantially attenuated the effects of Apo2L on glioma cell numbers at concentrations up to 1000 ng/ml (Fig. 5).

In view of the significant activity of Apo2L against glioma cell lines *in vitro* and the selective toxicity observed in comparison with non-neoplastic astrocytes, we examined the activity of

this agent *in vivo* using a human U87 glioma xenograft model. Kaplan-Meier survival curves for the control animals and those treated with various concentrations of Apo2L are shown in Fig. 6. Whereas median survival in control animals was  $27 \pm 1.7$  days, median survivals for animals receiving Apo2L/TRAIL at doses of 0.3, 1, 3, 10, and 30 mg/kg/d for a 7-day period were each  $>50$  days after tumor implantation ( $55 \pm 2.5$  days,  $58 \pm 2.5$  days,  $60 \pm 4.5$  days,  $67 \pm 15$  days, and  $>120$  days, respectively). All of the four animals in the 0.3-mg/kg/d group survived between 53 and 59 days after tumor implantation, whereas the four animals in the 1-mg/kg/d group survived between 56 and 62 days. In the 3-mg/kg/d group, survival ranged between 55 and 65 days. In the 10-mg/kg/d group, one animal died at 48 days without obvious signs of intracranial tumor; the other three animals survived between 62 and 89 days. All of the animals in the 30-mg/kg/d group exhibited long-term ( $>120$  day) survival without evidence of tumor or signs of toxicity. Differences between survival of control animals and those receiving Apo2L/TRAIL were statistically significant ( $P < 0.001$ , Student's *t* test for survival duration;  $P < 0.01$ ,

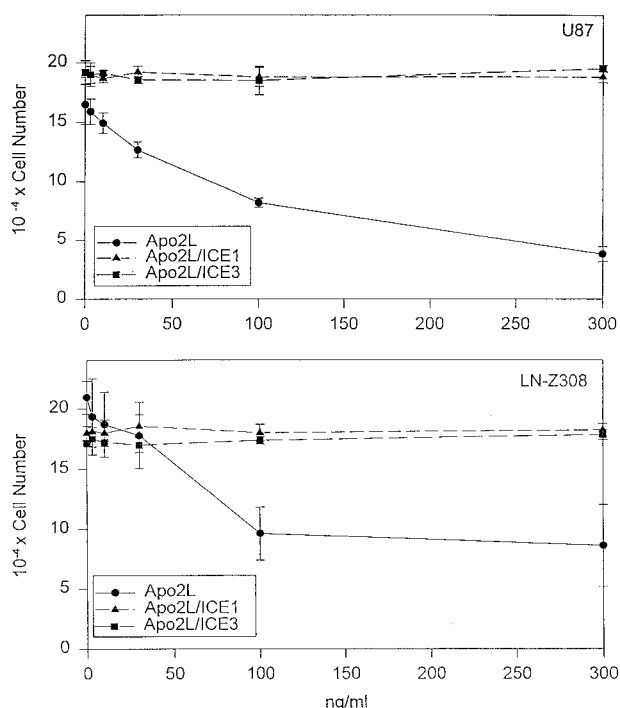


Fig. 5 Effect of caspase inhibitors on the activity of Apo2L. After pretreatment with Ac-YVAD-CMK or Ac-DEVD-CHO, U87 and LN-Z308 glioma cells were treated with Apo2L, and cell numbers were assessed as in Fig. 1.

rank-sum test for differences in survival curves). Differences in survival duration between the 30-mg/kg/d group and the groups that received lower doses were also statistically significant ( $P < 0.01$ ). On microscopic examination, the brains of long-term survivors showed no evidence of either residual tumor or other histological abnormalities.

## DISCUSSION

Although apoptosis functions to eliminate irreparably damaged or potentially deleterious cells (38–40), neoplastic cells often are resistant to the normal molecular triggers for apoptosis, such as DNA damage and mitogen depletion. Because death receptors such as TNFR1 and Fas/Apo1 directly stimulate the caspase cascade leading to apoptosis, selective targeting of such receptors provides a potential strategy for destroying tumor cells, which may be particularly useful for neoplasms such as malignant glioma, that are largely refractory to conventional therapeutic modalities (3, 15). Unlike radiotherapy or conventional chemotherapy, direct stimulation of death signaling induces killing in a p53-independent fashion, thus circumventing the obstacle provided by *TP53* mutations in a significant percentage of tumors (18). Unfortunately, early efforts to stimulate TNFR1 *in vivo* using systemic TNF infusion produced a severe inflammatory response resembling septic shock, which was thought to be attributable to activation of pro-inflammatory transcription factors, such as nuclear factor  $\kappa$ B, in macrophages and endothelial cells (16). Stimulation of Fas/Apo1 using an antibody capable of cross-linking the receptor was also shown to

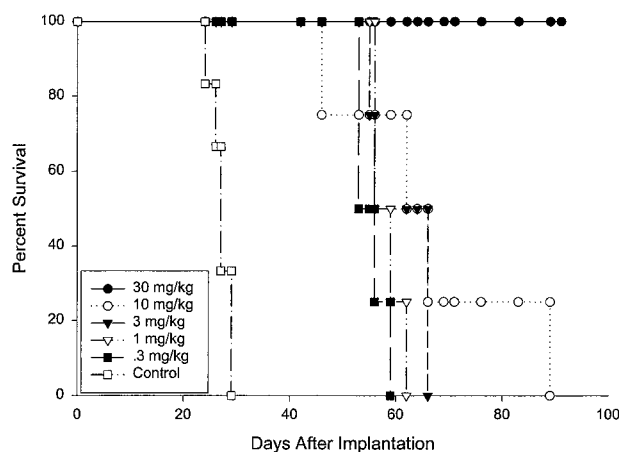


Fig. 6 Effect of Apo2L on survival of nude mice after intracranial implantation of U87 glioma cells. Kaplan-Meier survival curves for control animals and those treated with various concentrations of Apo2L are shown. Differences between control and each of the Apo2L-treated groups were statistically significant ( $P < 0.001$ ) as were the differences between the survival of animals in the 30 mg/kg/day-group and those receiving lower doses.

be lethal in murine models, possibly because of the high levels of Fas/Apo1 expression in normal cells, such as hepatocytes (19, 20).

Recent studies (24–29) have identified additional cell death receptors that may provide much more selective targets for tumor killing, namely DR4 and DR5 (Apo2). Although these receptors are ubiquitously expressed, they appear to mediate selective killing of certain tumor cells *versus* normal cells upon binding of their ligand, Apo2L/TRAIL. One mechanism that may contribute to this selectivity is the existence of “decoy” receptors for Apo2L (25–27, 29–32), such as DcR1 and DcR2. These receptors are expressed in many normal tissues, in which they may prevent induction of apoptotic signaling by Apo2L/TRAIL. In contrast, several types of neoplastic cells, including many glioma cell lines (34), express low or undetectable levels of these decoy receptors, which may render such cells selectively vulnerable to Apo2L/TRAIL-mediated toxicity compared with normal cells. An additional mechanism that may regulate sensitivity to Apo2L involves expression of the c-FLIP protein, which inhibits apoptotic signaling by death receptors (41).

In the current study, we explored the efficacy of recombinant soluble Apo2L/TRAIL as a means for producing selective toxicity in glioma cells *versus* non-neoplastic astrocytes. These studies showed a median effective concentration for decreasing cell numbers that ranged from 30 to 100 ng/ml (0.5–1.7 nM) in four glioma cell lines, including one with mutated *TP53* and one with deleted *TP53*. Although there was some difference between the cell lines in the magnitude of the effects observed, this does not appear to relate directly to their pattern of expression of the agonist receptors DR4 and DR5 or the decoy receptors DcR1 and DcR2 (34).

In contrast to the significant effects observed in glioma-derived cell lines, 100-fold higher concentrations of Apo2L produced no significant decrease in the numbers of non-neoplastic astrocytes. These results contrast with those of

Walczak *et al.* (42), in which some toxicity was observed in non-neoplastic astrocytes treated with a fusion protein that contained the extracellular sequence of Apo2L/TRAIL (amino acids 95–281) linked to a trimerizing leucine zipper expressed in Chinese hamster ovary cells. However, a recent report (35) involving the same TRAIL preparation used in the current study, specifically a homotrimeric soluble TRAIL containing a shorter extracellular domain (amino acids 114–281) and no heterologous sequences, observed no toxicity in non-neoplastic astrocytes at concentrations as high as 1  $\mu\text{g/ml}$ .

Our observation of a concentration-dependent cytotoxic effect against glioma cells was confirmed using a series of clonogenic assays, which demonstrated a steep drop-off in colony-forming activity at nanomolar concentrations of the ligand. The induction of cytotoxicity correlated with the detection of DNA fragmentation as assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling assay, in agreement with recent observations (34). Inhibitors of caspase activity substantially diminished the effects of Apo2L on glioma cell survival, supporting the importance of these proteases in mediating Apo2L-induced glioma cell killing *in vitro*.

Because it is conceivable that the differential sensitivity of glioma-derived cell lines *versus* non-neoplastic astrocytes to Apo2L/TRAIL could simply result from inherent differences in their relative abilities to propagate and survive *in vitro*, it was recognized that a more therapeutically relevant measure of the potential utility of this agent would be its ability to inhibit tumor growth *in vivo*. An additional caveat for brain tumor therapeutics is that agents that are effective against systemic tumors may not necessarily be useful for the treatment of central nervous system tumors, because the blood-brain barrier may prevent adequate concentrations of a systemically administered drug from reaching the tumor. In that context, the demonstration of significant efficacy for Apo2L/TRAIL in an *in vivo* glioma model system is particularly striking. A single 7-day course of Apo2L/TRAIL, at doses ranging from 0.3 to 30 mg/kg/day, produced a significant, dose-dependent prolongation of survival duration. Mean survival ranged from  $55 \pm 2.5$  days in animals that received 0.3 mg/kg/day to  $>120$  days in all of the animals that received 30 mg/kg/day, as compared with only  $27 \pm 1.5$  days in vehicle-treated controls. None of the animals at the highest dose level showed evidence of weight loss or neurological impairment, and histological examination of the brains in several of the long-term survivors showed no evidence of either tumor or other histological abnormalities. The lack of obvious toxicity at these dose levels is consistent with recent toxicology studies in mice using a mammary carcinoma model (42) and a colon xenograft model (35). In addition, detailed studies in nonhuman primates have shown no histological evidence of toxicity within brain, lung, liver, heart, kidney, ovary, testis, spleen, bone marrow, or lymph nodes; no alteration of leukocyte counts, liver enzyme activity, coagulation profiles, serum chemistry, or urine analysis; and no evidence of anti-Apo2L/TRAIL antibody titers (35).

In conclusion, we observed potent induction of apoptosis by recombinant soluble Apo2L/TRAIL in a series of four glioma cell lines. The toxic effects of this ligand were selective for glioma cells *versus* non-neoplastic astrocytes. Apo2L/TRAIL treatment caused striking inhibition of tumor growth in a pre-

clinical intracranial glioma model, with apparent “cure” of all of the animals treated at the highest dose levels without obvious systemic toxicity. The observation that systemic administration of Apo2L/TRAIL was effective in eliminating the growth of this otherwise fatal central nervous system tumor indicates that this may be a relevant agent for the therapeutic management of malignant gliomas that merits further evaluation.

## ACKNOWLEDGMENTS

We thank Roger C. Pai, Susan Leung, Zahra Shahrokh, and Genentech's Apo2L project team for providing purified Apo2L.

## REFERENCES

- Kerr, J. F., Winterford, C. M., and Harmon, B. V. Apoptosis. Its significance in cancer and cancer therapy. *Cancer (Phila.)*, 73: 2013–2026, 1994.
- Lowe, S., Bodis, S., McClatchy, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. p53 status and the efficacy of cancer therapy *in vivo*. *Science (Wash. DC)*, 266: 807–810, 1994.
- Devaux, B. C., O'Fallon, J. R., and Kelly, P. J. Resection, biopsy, and survival in malignant glial neoplasms. A retrospective study of clinical parameters, therapy, and outcome. *J. Neurosurg.*, 78: 767–775, 1993.
- Bennett, M., Macdonald, K., Chan, S-W., Luzio, J. P., Simari, R., and Weissberg, P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science (Wash. DC)*, 282: 290–293, 1998.
- Sheikh, M. S., Burns, T. F., Huang, Y., Wu, G. S., Amundson, S., Brooks, K. S., Fornace, A. J., Jr., and El-Deiry, W. S. p53-dependent, and -independent regulation of the death receptor *KILLER/DR5* gene expression in response to genotoxic stress and tumor necrosis factor  $\alpha$ . *Cancer Res.*, 58: 1593–1598, 1998.
- Pollack, I. F., Hamilton, R. L., Finkelstein, S. D., Campbell, J. W., Martinez, A. J., Sherwin, R., Bozik, M. E., and Gollin, S. M. The relationship between *TP53* mutations and overexpression of p53 and prognosis in malignant gliomas of childhood. *Cancer Res.*, 57: 304–309, 1997.
- Sidransky, D., Mikkelsen, T., Schechheimer, K., Rosenblum, M. L., Cavenee, W., and Vogelstein, B. Clonal expansion of p53 mutant cells is associated with brain tumor progression. *Nature (Lond.)*, 355: 846–847, 1992.
- Maxwell, M., Naber, S. P., Wolfe, H. J., Galanopoulos, T., Hedley-Whyte, E. T., Black, P. M., and Antoniades, N. H. Co-expression of platelet-derived growth factor (PDGF) and PDGF-receptor genes by primary human astrocytomas may contribute to their development and maintenance. *J. Clin. Investig.*, 86: 131–140, 1990.
- Nister, M., Claesson-Welch, L., Eriksson, A., Heldin, C-H., and Westermark, B. Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines. *J. Biol. Chem.*, 266: 16755–16763, 1991.
- Morrison, R. S., Yamaguchi, F., Saya, H., Bruner, J. M., Yahanda, A. M., Donehower, L. A., and Berger, M. Basic fibroblast growth factor and fibroblast growth factor receptor I are implicated in the growth of human astrocytomas. *J. Neuro-oncol.*, 18: 207–216, 1994.
- Gross, J. L., Herblin, W. F., Dusak, B. A., Czerniak, P., Diamond, M. D., Sun, T., Eidsvoog, K., Dexter, D. L., and Yayon, A. Effects of modulation of basic fibroblast growth factor on tumor growth *in vivo*. *J. Natl. Cancer Inst. (Bethesda)*, 85: 121–131, 1993.
- Glick, R. P., Unterman, T. G., and Hollis, R. Radioimmunoassay of insulin-like growth factors in cyst fluid of central nervous system tumors. *J. Neurosurg.*, 74: 972–978, 1991.
- Gammeltoft, S., Ballotti, R., Kowalski, A., Westermark, B., and van Obberghen, E. Expression of two types of receptor for insulin-like growth factors in human malignant glioma. *Cancer Res.*, 48: 3910–3918, 1988.
- Wong, A. J., Bigner, S. H., Bigner, D. D., Kinzler, K. W., Hamilton, S. R., and Vogelstein, B. Increased expression of the epidermal growth

- factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc. Natl. Acad. Sci. USA*, *84*: 6899–8903, 1987.
15. Campbell, J., Pollack, I. F., Shultz, B. L., and Martinez, A. J. Prognostic factors in the management of pediatric malignant gliomas. *Neurosurgery*, *38*: 258–264, 1996.
  16. Ashkenazi, A., and Dixit, V. M. Death receptors: signaling and modulation. *Science (Wash. DC)*, *281*: 1305–1308, 1998.
  17. Smith, C. A., Farrah, T., and Goodwin, R. G. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell*, *76*: 959–962, 1994.
  18. Yount, G. L., Levine, K. S., Kuriyama, H., Haas-Kogan, D. A., and Israel, M. A. Fas (APO-1/CD95) signaling pathway is intact in radioreistant human glioma cells. *Cancer Res.*, *59*: 1362–1365, 1999.
  19. Nagata, S. Apoptosis by death factor. *Cell*, *88*: 355–365, 1997.
  20. Ogasawara, J., Watanabe, F. R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. Lethal effect of the anti-Fas antibody in mice. *Nature (Lond.)*, *364*: 806–809, 1993.
  21. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., *et al.* Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*, *3*: 673–682, 1995.
  22. Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., and Ashkenazi, A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J. Biol. Chem.*, *271*: 12687–12690, 1996.
  23. Marsters, S. A., Pitti, R. M., Donahue, C. J., Ruppert, S., Bauer, K. D., and Ashkenazi, A. Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. *Curr. Biol.*, *6*: 750–752, 1996.
  24. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M. The receptor for the cytotoxic ligand TRAIL. *Science (Wash. DC)*, *276*: 111–113, 1997.
  25. Pan, G., Ni, J., Wei, Y-F., Yu, G-L., Gentz, R., and Dixit, V. M. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science (Wash. DC)*, *277*: 815–818, 1997.
  26. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science (Wash. DC)*, *277*: 818–821, 1997.
  27. Schneider, P., Bodmer, J. L., Thome, M., Hofmann, K., Holler, N., and Tschopp, J. Characterization of two receptors for TRAIL. *FEBS Lett.*, *416*: 329–334, 1997.
  28. Wu, G. S., Burns, T. F., McDonald, E. R., III, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and El-Deiry, W. S. KILLER/DR5 is a DNA damage-inducible p53-regulated cell death receptor gene. *Nat. Genet.*, *17*: 141–143, 1997.
  29. MacFarlane, M., Ahmad, M., Srinivasula, S. M., Fernandes-Alnemri, T., Cohen, G. M., and Alnemri, E. S. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J. Biol. Chem.*, *272*: 25417–25420, 1997.
  30. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr. Biol.*, *7*: 1003–1006, 1997.
  31. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. The novel receptor TRAIL-R4 induces NF- $\kappa$ B and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity*, *7*: 813–820, 1997.
  32. Pan, G., Ni, J., Yu, G. L., Wei, Y. F., and Dixit, V. M. TRUND, a new member of the TRAIL receptor family that antagonizes TRAIL signaling. *FEBS Lett.*, *424*: 41–45, 1998.
  33. Keane, M. M., Ettenberg, S. A., Nau, M. M., Russell, E. K., and Lipkowitz, S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res.*, *59*: 734–741, 1999.
  34. Rieger, J., Naumann, U., Glaser, T., Ashkenazi, A., and Weller, M. Apo2 ligand: a novel lethal weapon against malignant glioma? *FEBS Lett.*, *427*: 124–128, 1998.
  35. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtry, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokhi, Z., and Schwall, R. H. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J. Clin. Investig.*, *104*: 155–162, 1999.
  36. Enari, M., Talianian, R. V., Wong, W. W., and Nagata, S. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature (Lond.)*, *380*: 723–726, 1996.
  37. Martuza, R. L., Mallick, A., Markert, J. M., Ruffner, K. L., and Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science (Wash. DC)*, *252*: 854–856, 1991.
  38. Thornberry, N. A., and Lazebnik, Y. Caspases: enemies within. *Science (Wash. DC)*, *281*: 1312–1316, 1998.
  39. Jacobson, M. D., Weil, M., and Raff, C. Programmed cell death in animal development. *Cell*, *88*: 347–354, 1997.
  40. Steller, H. Mechanisms and genes of cellular suicide. *Science (Wash. DC)*, *267*: 1445–1449, 1995.
  41. Griffith, T. S., Chin, W. A., Jackson, G. C., Lynch, D. H., and Kubin, M. Z. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J. Immunol.*, *161*: 2833–2840, 1998.
  42. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Schuh, J. C. L., and Lynch, D. H. Tumorcidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat. Med.*, *5*: 157–163, 1999.



# Clinical Cancer Research

## Direct Stimulation of Apoptotic Signaling by Soluble Apo2L/Tumor Necrosis Factor-related Apoptosis-inducing Ligand Leads to Selective Killing of Glioma Cells

Ian F. Pollack, Melanie Erff and Avi Ashkenazi

*Clin Cancer Res* 2001;7:1362-1369.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/7/5/1362>

**Cited articles** This article cites 41 articles, 19 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/7/5/1362.full#ref-list-1>

**Citing articles** This article has been cited by 15 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/7/5/1362.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://clincancerres.aacrjournals.org/content/7/5/1362>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.