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TRAIL-induced apoptosis in gliomas is enhanced by Akt-inhibition and is independent of JNK activation*

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Abstract

Patients with malignant gliomas have a poor prognosis and new treatment paradigms are needed against this disease. TRAIL/Apo2L selectively induces apoptosis in malignant cells sparing normal cells and is hence of interest as a potential therapeutic agent against gliomas. To determine the factors that modulate sensitivity to TRAIL, we examined the differences in TRAIL-activated signaling pathways in glioma cells with variable sensitivities to the agent. Apoptosis in response to TRAIL was unrelated to DR5 expression or endogenous p53 status in a panel of 8 glioma cell lines. TRAIL activated the extrinsic (cleavage of caspase-8, caspase-3 and PARP) and mitochondrial apoptotic pathways and reduced FLIP levels. It also induced caspase-dependent JNK activation, which did not influence TRAIL-induced apoptosis. Because the pro-survival PI3K/Akt pathway is highly relevant to gliomas, we assessed whether Akt could protect against TRAIL-induced apoptosis. Pretreatment with SH-6, a novel Akt inhibitor, enhanced TRAIL-induced apoptosis, suggesting a protective role for Akt. Conversely, TRAIL induced caspase-dependent cleavage of Akt neutralizing its anti-apoptotic effects. These results demonstrate that TRAIL-induced apoptosis in gliomas involves both activation of death pathways and downregulation of survival pathways. Additional studies are warranted to determine the therapeutic potential of TRAIL against gliomas.

Keywords

Akt; apoptosis; death receptor; glioma; signal transduction; TRAIL

Introduction

TRAIL/Apo2L, a member of the TNF family, has demonstrated selectivity against malignant cells and is of therapeutic interest against nonmetastatic locoregional malignancies such as gliomas. It interacts with the death receptors, DR4 and DR5 (KILLER), and initiates a caspase-mediated cascade culminating in apoptosis.¹ This process involves recruitment of an adaptor protein, likely FADD, and subsequent activation of caspase-8 and caspase-3.²⁻⁴ Both the extrinsic and intrinsic apoptotic pathways are activated downstream of TRAIL providing a complex signaling pathway that allows several levels of regulation.⁵⁻⁷ TRAIL also associates with the decoy receptors, DcR1 and DcR2, the levels

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of expression of which were initially thought to determine cellular sensitivity to TRAIL;^{8,9} it has subsequently been shown, however, that receptor levels are not primary determinants of cellular sensitivity.^{10,11}

Tumor cells are selectively sensitive to TRAIL-induced apoptosis, whereas most normal cells are resistant to its effects.^{12–14} Preclinical studies examining the efficacy and toxicity of TRAIL in xenograft models have shown its potential as a therapeutic agent against malignancies.^{15,16} These data may be particularly relevant to nonmetastatic and regionally infiltrative tumors such as malignant gliomas, which may be amenable to local therapeutic approaches. Additionally, agents such as TRAIL that can selectively target tumor cells without causing toxicity to the surrounding normal tissue are of interest in clinical approaches to this disease.

TRAIL induced signaling is tightly regulated at various levels at and downstream of the receptors. Such complex regulatory mechanisms are believed to permit cross talk between the apoptotic and survival mechanisms allowing tight regulation of TRAIL-induced signaling. The role of the molecules such as Akt, which are involved in resistance to the apoptotic process, has been evaluated previously using overexpression paradigms. However, their relevance to TRAIL signaling and cellular sensitivity in gliomas in an endogenous setting has not been adequately studied. Similarly, JNK activation has been implicated with both facilitation and resistance to apoptosis signals and its significance to TRAIL-induced apoptosis in gliomas is uncertain. To address these gaps, we examined the role of Akt and JNK pathways, which are relevant to cellular survival, and stress induced pathways respectively in glioma cells with variable sensitivities to TRAIL.

Materials and methods

Cell lines

All cell lines used in the study were derived from human malignant gliomas. D54MG cells were a generous gift from Dr. Darrell Bigner (Duke University, Durham). The U251MG cell line was obtained from the National Cancer Institute. U87MG, U343, U373, A172, LN229 and T98G cells were purchased from American Tissue Type Collection (ATCC). The cells were maintained in DMEM-F12 (1:1,v/v) medium (Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal calf serum, at 37°C in a humidified atmosphere containing 5% CO₂.

Reagents and plasmids

Recombinant soluble TRAIL (His-tagged) was obtained commercially (Biomol, Plymouth Meeting, PA). To study the effect of caspase inhibition, the cells were preincubated with 100 μ M of z-DEVD-fmk or z-IETD-fmk (Oncogene Research Products, San Diego, CA) for 24 h prior to addition of TRAIL. To inhibit JNK activity, cells were treated with Forskolin (Sigma Chemical Co., St. Louis, MO) at a concentration of 100 μ M for 6 h prior to treatment with TRAIL. Additional experiments were performed using the fusion product D-JNK1, a cell permeable JNK inhibitor, which utilizes the ability of HIV-TAT protein to achieve cell permeability (Alexis Biochemicals, San Diego, CA). The novel Akt-specific inhibitor, SH-6, which does not inhibit PI-3-kinase nor interfere with activation of PDK-1 was obtained commercially (Alexis Biochemicals).¹⁷ The plasmids encoding wt Akt (p-FLAG-hAkt1) and the caspase noncleavable Akt mutants pFLAG-Akt1(D108A), pFLAG-Akt1(D119A) and the double mutant p-FLAG-Akt1 (D108A/D119A) were kindly provided by Drs. T. Tsuruo and N. Fujita (University of Tokyo, Tokyo, Japan) and have been previously described.¹⁸

XTT assay

The cells were plated in triplicate to a final volume of 200 μ L/well at a density of 2×10^3 cells/well in a 96-well microtiter plate. The cells were exposed to TRAIL (1 μ g/ml) for 0, 24, and 48 h. The XTT assay was performed according to the manufacturer's specifications (Roche Applied Science, Indianapolis, IN). Briefly, 50 μ L XTT reagent mixed with the electron-coupling reagent was added to the wells and the plate was incubated for 6 h at 37°C. Spectrophotometric absorbance was quantitated at a 490 nm wavelength using a microtiter plate reader.

Cell transfection

D54MG cells were harvested at 50–60% confluence and plated at a density of 2×10^6 cells per dish in 60 mm dishes in DMEM-F12 medium with 15% FBS. After the cells had attached, they were transfected with 1 μ g of pEGFP control vector, pFLAG-Akt, pFLAG-Akt (108A), pFLAG-Akt (119A) or p-mt Akt (108A/119A) using Fugene 6 according to the manufacturer's instructions. Transfection efficiency was verified by exogenous expression of a plasmid that generated EGFP.

Flow cytometry

Cells were plated at a density of 10^5 cells/dish in 60 mm dishes. Soluble TRAIL was added 24 h after plating the cells. The cells were harvested at 24, 72, and 96 h, and fixed in 70% ethanol. The cells were subsequently treated with propidium iodide (50 mg/ml) and RNase (20 mg/ml) for 15 min, prior to flow cytometry using an EPICS II flow cytometer (Coulter Corporation, Hialeah, FL) equipped with an air-cooled argon ion laser emitting at a wavelength of 488 nm at 15 mw. A minimum of 10,000 events was analyzed per sample using the Coulter cytological program.

TUNEL assay

A terminal deoxynucleotidyl transferase (TdT) assay was performed using the APO-DIRECT™ kit according to the protocol recommended by the manufacturer (Phoenix Flow Systems, Inc., San Diego, CA). Briefly, 10^5 cells plated in 60 mm dishes were harvested after treatment with TRAIL and processed for flowcytometric studies. The cells were fixed using freshly prepared 1% paraformaldehyde in PBS for 15 min on ice and incubated overnight at room temperature in a staining solution containing TdT enzyme, fluorescein-dUTP, and TdT reaction buffer. Following this, the cells were rinsed twice and resuspended in a solution of propidium iodide/RNase-A prior to flowcytometric analysis.

Western blot analysis

Equal amounts of protein from TRAIL-treated and untreated cells were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH). The membrane was blocked using Blotto-Tween (5% non-fat dry milk, 0.05% Tween, 0.9% NaCl, and 50 mM Tris, pH 7.5). The following antibodies were used to detect the respective protein: caspase-8 mouse monoclonal IgG Ab-3 (Oncogene Research Products, San Diego, CA), cpp32 rabbit polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:2000 dilution, PARP mouse monoclonal IgG (PharMingen, San Diego, CA) at a 1:200 dilution, Akt rabbit polyclonal IgG, phospho-Akt (Ser-473) rabbit polyclonal IgG (Cell Signaling Technology, Beverly, MA) and anti-human c-FLIP NF6 (kindly provided by Dr. Marcus Peter, University of Chicago, IL).¹⁹ Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse antibodies (Amersham Corp., Arlington Heights, IL) were used as secondary antibodies. Actin levels were determined as a loading control using a mouse monoclonal anti-human actin antibody (Amersham Corp.).

Cytochrome C release studies

To assess cyt C release from the mitochondria, cells were washed in PBS and incubated for 30 min on ice in lysis buffer (68 mmol/L sucrose, 200 mmol/L mannitol, 50 mmol/L KCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L DTT, and 1x Complete protease inhibitor [Roche]). The cells were subsequently homogenized with an ultrasound sonicator and centrifuged at 4°C (800g) to remove nuclei, unbroken cells, and debris. The supernatant was centrifuged at 14 000g for 15 min. The supernatant (cytoplasmic fraction) and pellets (mitochondria) were stored at -70°C for immunoblot analysis. The cytoplasmic fraction was analyzed for the presence of cyt C, indicating its release from the mitochondria.

JNK assay

Cells were treated with 1 µg/ml TRAIL for the duration indicated and total protein was collected as described.^{20,21} JNK activity was immunoprecipitated by rabbit anti-JNK antibodies. The precipitates were washed twice with lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6) and twice with kinase buffer (20 mM MOPS, pH 7.2; 2 mM EGTA, 10 µM MgCl₂, 1 mM dithiothreitol, 0.1% triton X-100 and 0.1 mM Na₃VO₄). The pellets were mixed with 2 mg GST-jun-(1-331), 15 mM ATP and 20 mCi of [³² P]ATP in 30 ml of kinase buffer for 20 min at 30°C. The reaction was terminated with an equal volume of Laemmli sample buffer and the products resolved with 10% SDS-PAGE and autoradiography. The relative kinase activities were normalized to the amounts of immunoprecipitated JNK assayed by immunoblotting and visualized by chemiluminescence.

Results

TRAIL activates the direct and indirect apoptotic pathways in glioma cells independent of DR5 expression or p53 status

We studied the effects of TRAIL on a panel of 8 glioma cell lines with varied p53 status (cells with wt p53, D54MG, U87MG, LN229 and U343; cells with mutant p53, U251, T98G, and U373). Of these cells, U87, LN229 and U373 cells were resistant to TRAIL-induced apoptosis whereas the remainder of the cells showed variable susceptibility to the agent, which was independent of their p53 status (Figure 1A). We also assessed the DR5 expression level to determine its relevance to TRAIL-sensitivity and the endogenous p53 status (data not shown). No correlation was found between TRAIL sensitivity and levels of DR5 expression or the endogenous p53 status. TRAIL activated caspase-8 in D54MG but not in U87MG cells. Pretreatment with the caspase-8 inhibitor, z-IETD-fmk, prevented caspase-8 activation (Figure 1B) and abrogated TRAIL-induced cell death. TRAIL treatment also resulted in the expected downstream events, including caspase-3 activation and cleavage of poly-ADP-ribose polymerase (PARP) in D54MG cells (data not shown). Consistent with the role of caspase-3 acting downstream of caspase-8 in the death receptor-activated pathway, inhibition of caspase-3 processing using z-DEVD-fmk did not abrogate activation of caspase-8, but inhibited TRAIL-induced apoptosis.

Coincident with caspase-8 activation, TRAIL treatment resulted in cleavage of BID within 3 h of treatment in D54MG but not U87MG cells with the concurrent release of cyt C from the mitochondria (Figure 2); inhibition of caspase-8 using z-IETD-fmk prevented TRAIL-induced BID cleavage and release of cyt C. Interestingly, when caspase-3 processing was inhibited by z-DEVD-fmk, neither cleavage of BID nor the subsequent release of cyt C was evident in the TRAIL-sensitive D54MG cell line, although caspase-8 processing occurred after treatment with z-DEVD-fmk). This finding suggests that caspase-3 could play a role in caspase-8-induced cleavage of BID in glioma cells or induce the release of cyt C by itself.

TRAIL treatment results in a reduced FLIP_L level in sensitive cells

Intracellular regulation of sensitivity to TRAIL-induced apoptosis occurs at several levels in the submembrane-signaling pathway. FLIP_L (FLIP, hereafter) can inter-act with caspase-8 and inhibit apoptosis. We hypothesized that downregulation of FLIP would be necessary for unhindered transmission of the apoptotic signal in TRAIL-sensitive cells. Treatment of D54MG, U87MG, LN229 and normal human astrocytes (NHA) cells using TRAIL with the TRAIL-sensitive 293 cells as a control, resulted in reduced levels of unprocessed FLIP in D54MG but not in LN229, U87MG or NHA cells, which corresponded with the individual sensitivities of these cells to TRAIL (Figure 3). In the TRAIL sensitive D54 cells, the decrease in FLIP levels was accompanied by the appearance of an additional 44 kD band which is likely a cleavage product in response to TRAIL treatment.

TRAIL downregulates Akt levels by caspase-dependent cleavage

Due to the frequent deletion of the *MMAC1/PTEN* gene in malignant gliomas, the PI3 kinase/Akt pathway is up-regulated in these tumors and is relevant to their growth and proliferation. The PI3K/Akt pathway has also been implicated in the resistance to TRAIL-induced apoptosis in prostate cancer cells, suggesting that this mechanism may be relevant in gliomas; however, whether this finding is relevant to other cell types remains controversial.^{22–24} To determine if TRAIL exerts its effect on cell viability by inhibiting signaling pathways involved in glioma cell survival, we assessed the levels of phosphorylated Akt in D54MG and U87MG cells. U87MG cells lack a functional *MMAC1/PTEN* gene and constitutively overexpress Akt, which is phosphorylated at both the Ser-473 and Thr-308 positions.²⁵ D54MG cells also showed a constitutive overexpression of phosphorylated Akt. Upon treatment with TRAIL, the levels of phosphorylated Akt diminished in D54MG but not in U87MG cells, suggesting that this effect correlated with the sensitivity of the cells to the effects of TRAIL. Further analysis showed that the decrease in phosphorylated Akt reflected a decrease in total Akt levels (Figure 4A). A cleaved product that corresponded with a previously described Akt cleavage product was seen in the TRAIL-treated samples. The reduction in Akt levels in response to TRAIL treatment in D54MG cells was abrogated by caspase inhibitors, suggesting that Akt was cleaved by caspases (Figure 4B).

TRAIL-induced Akt cleavage could be independent from caspase-3

Previous reports have suggested that Akt is cleaved in response to caspase-3 activation and that specific aspartate residues (Asp108 and Asp119) are targeted by this caspase, resulting in Akt degradation.^{18,26} Given the central role for activated caspase-3 in TRAIL-induced apoptosis, we asked if the Akt degradation seen in glioma cells in response to TRAIL was specifically mediated by caspase-3. D54MG cells, transfected with the plasmids encoding wt Akt (pFLAG-hAkt1) or the caspase-3-noncleavable Akt mutants, pFLAG-Akt (D108A), pFLAG-Akt (D119A), or the double mutants pFLAG-Akt1(D108A, D119A) which contain alanine substitutions at the corresponding aspartate sites, were exposed to TRAIL for 6 h and the levels of total Akt were determined using anti-Akt antibody by western blot analysis. TRAIL treatment resulted in degradation of the caspase-3-noncleavable Akt mutants to the same degree as the overexpressed wt-Akt and the endogenous Akt, suggesting that caspase-3 did not play a significant role in Akt cleavage in gliomas (Figure 5A). Overexpression of the exogenous mutant Akt forms, confirmed by assessing expression of FLAG-tagged proteins using an anti-FLAG M2 antibody and by EGFP expression as a measure of transfection efficiency (~30%), did not inhibit TRAIL-induced apoptosis as assessed by the sub-G1 fraction compared with cells overexpressing wt Akt (Figure 5B). Robust expression of the FLAG tagged protein was detected in the cells transfected expressing FLAG-tagged proteins, which constituted nearly half the total cellular Akt. These results, combined with

the finding that caspase inhibitors can abrogate Akt cleavage in response to TRAIL, strongly suggest that caspases other than caspase-3 are possibly involved in Akt cleavage.

Inhibition of Akt in gliomas enhances susceptibility to TRAIL

Akt overexpression has been shown to protect against TRAIL-induced apoptosis in some cell types.²² Also, inhibition of the PI3-kinase/Akt pathway has been shown to increase susceptibility to TRAIL in certain cell types.²⁴ Due to the possibility that Akt may similarly protect against TRAIL-induced apoptosis in gliomas, we hypothesized that inhibition of endogenous Akt activity could enhance TRAIL-induced apoptosis. Because Wortmannin and LY294002 are not specific inhibitors of Akt and could induce diverse effects via inhibition of PI3-kinase and its other downstream targets, we utilized SH-6, a novel cell-permeable agent that specifically inhibits Akt without effects on PI3-kinase or PDK1.¹⁷ Pretreatment of D54MG cells with SH-6 (10 or 20 μ M) followed by addition of TRAIL (1 μ g/ml) resulted in dose-dependent enhancement in degree of apoptosis compared with cells exposed to TRAIL alone (Figure 6A). SH-6 did not by itself induce apoptosis in D54MG cells, suggesting that the enhancement of apoptosis was due to the effect of TRAIL in the context of SH-6 induced decreased Akt activity and not due to an independent activity of SH-6. SH-6-treated cells showed a slightly reduced level of phosphorylated (Ser-473) Akt but not total Akt (Figure 6B). Consistent with the expected inhibition of downstream effects of Akt, SH-6-treated cells showed a decrease in FLIP levels compared to control cells. These results suggest a protective role for Akt against TRAIL-induced apoptosis. Conversely, abrogation of Akt activity, such as by cleavage and degradation of the protein, could enhance the effectiveness of TRAIL-induced apoptosis.

TRAIL activates JNK via caspase-dependent and caspase-independent pathways

Members of the TNF superfamily such as TNF- α and Fas are known to activate the stress-activated protein kinase, JNK.²⁷ To determine if the JNK signaling pathway is activated during TRAIL-induced apoptosis in gliomas, D54MG and U87MG cells were exposed to TRAIL. The ability of the total cellular protein lysates to phosphorylate the substrate, c-jun, was studied in an *in vitro* kinase assay used to measure JNK activation. In the TRAIL-sensitive D54MG cell line JNK activation was seen at 1 h after adding TRAIL, increasing to its maximum activity by 6 h (Figure 7).

JNK activation by caspase-dependent and caspase-independent mechanisms has been demonstrated in certain cell types following ligation of the death receptors.^{4,28} To determine a possible regulatory role for caspases in JNK activation in glioma cells, D54MG cells were exposed to the caspase inhibitors, z-IETD-fmk and z-DEVD-fmk, before being exposed to TRAIL for 6 h. When caspase-8 and caspase-3 were inhibited, JNK activity was also inhibited. In contrast, when U87MG cells were exposed to TRAIL, JNK was only minimally activated (data not shown). These results correlate well with the lack of caspase activation and resistance to TRAIL-induced death observed in these cells. Taken together, our data suggest that caspases have a role in the activation of the JNK signaling pathway in response to TRAIL in glioma cells.

TRAIL-induced JNK activation does not correlate with induction of apoptosis

To determine if the JNK signaling pathway contributed to TRAIL-induced apoptosis in gliomas, we studied how inhibiting JNK activation affected the induction of apoptosis in the TRAIL-sensitive D54MG cells. D54MG cells were exposed to D-JNK1, a cell-permeable inhibitor of JNK, or to a control peptide D-TAT for 2 h prior to TRAIL treatment. As expected, cells exposed to D-TAT exhibited a robust activation of JNK in response to TRAIL with the cells undergoing apoptosis, as measured by the sub-G1 DNA content. When D54MG cells were incubated with D-JNK1 for 2 h and then subsequently exposed to

TRAIL, however, JNK activity was inhibited but the degree of cell death was not decreased compared with D54MG cells exposed to TRAIL alone or with those exposed to D-TAT and then to TRAIL (Figure 8A and B). These results suggest that the activation of JNK by TRAIL appeared to be independent of its ability to induce apoptosis. To ensure that the effect of JNK activation on apoptosis was not masked by the TRAIL concentration (1 μ g/ml) used in this study, D54MG cells were exposed to lower doses of TRAIL (100 ng/ml and 500 ng/ml) with or without prior treatment with D-JNKI and assessed for JNK activation and induction of apoptosis. JNK activation was seen even at the lowest TRAIL concentration used (100 ng/ml) (Figure 8C). Inhibition of JNK activation using D-JNKI did not affect the degree of TRAIL-induced apoptosis (Figure 8C, lower panel), confirming that JNK activation is not relevant to this effect in glioma cells in contrast with other malignant cell types.²⁸

Discussion

Ligation of TRAIL to its receptors activates a complex set of signaling pathways, which ultimately results in induction of apoptosis. Due to its crucial association with survival and death signals, p53 influences cellular responses to death signals such as Fas ligand and TNF in certain cell types.^{29,30} Abnormalities in p53 status, frequently observed in malignant gliomas, are associated with the resistance of malignant cells to apoptosis.^{31,32} In the case of TRAIL, previous studies demonstrated that *DR5*, a p53-inducible gene, is upregulated in response to ionizing radiation or DNA-damaging agents,³³ although how levels of endogenous p53 determine sensitivity to TRAIL is not known. TRAIL-induced apoptosis was evident in the glioma cells studied, but the susceptibility to apoptosis varied in different cell lines. The finding that sensitivity to TRAIL was independent of p53 status in glioma cells is particularly relevant to therapeutic considerations of TRAIL in the clinical setting where more than 50% of malignant gliomas exhibit p53 mutations and/or inactivation of the p53 pathway via alterations in *MDM2* or *p14/ARF*.³⁴ Our data also suggest that there is no correlation between levels of DR5 and endogenous p53 status or sensitivity to TRAIL in glioma cells.

In this study, caspase-8 was activated in TRAIL-sensitive glioma cells similar to previous reports in other cell types;^{12,35} inhibition of caspase-8 was sufficient to prevent apoptosis, confirming a primary role for this apical protease in TRAIL-induced apoptosis.³ Interestingly, partial caspase processing was seen in the TRAIL-resistant U87MG cells without activation of downstream apoptotic events, suggesting that mechanisms of resistance to TRAIL-induced apoptosis could, in part, act downstream of the apical caspases. Together, these results suggest that caspase-8 is the primary apical caspase involved in TRAIL-induced apoptosis in gliomas. TRAIL treatment also resulted in recruitment of the indirect apoptotic pathway as seen by the cleavage of Bid and release of cyt C from the mitochondrial membrane. Despite initial assertions regarding the specificity of commercially available tetrapeptide caspase inhibitors for individual caspases, more detailed analysis has shown that these chemicals can also inhibit other caspases although at a higher concentration than the target caspase. These findings have occasionally confounded the interpretation of caspase inhibition experiments. Data in this study suggests that z-DEVD fmk (a caspase 3 inhibitor whose aldehyde form has been shown to inhibit caspase 8 also) was unable to prevent caspase 8 processing compared with z-IETD-fmk in D54 cells (Figure 2) whereas both inhibitors prevented induction of apoptosis. These findings suggest that the level of inhibition by z-IETD-fmk was upstream of that by z-DEVD-fmk supporting the conclusions regarding the sequence of caspase activation in gliomas.

In addition to activating apoptotic signals, TRAIL also modulates the varied components of cell survival pathways via mechanisms that have not yet been fully delineated.

Overexpression of FLICE-inhibitory protein (FLIP), an endogenous caspase-8 inhibitor, has been shown to protect cells against TRAIL-induced apoptosis. FLIP in turn can be regulated via the PI3-kinase/Akt pathway; Akt downregulation results in decreased levels of FLIP.³⁶ This relationship suggests that FLIP's regulation via the PI3-kinase/Akt pathway could modulate TRAIL sensitivity at the level of caspase-8. Because TRAIL treatment in D54MG cells decreased the levels of FLIP expression, this mechanism of protection from death receptor-induced apoptosis is possibly inactivated in sensitive glioma cell lines, permitting unhindered caspase activation. In contrast, FLIP levels were unchanged in the TRAIL-resistant U87MG cells upon exposure to the ligand, although pre-treatment of these cells with cycloheximide, a protein synthesis inhibitor, resulted in rapid sensitization of the cells to TRAIL (data not shown). The reversal of resistance previously described in TRAIL-resistant cells after treatment with cycloheximide has been partly attributed to the fact that FLIP is a cycloheximide-sensitive component of the TRAIL pathway, which has been implicated in resistance to TRAIL.^{37,38}

The PI3-kinase/Akt cellular pathway is particularly relevant to proliferation and cell survival in malignant gliomas in which its constitutive activation results from the loss of function of *PTEN/MMAC1*. Activation of this pathway *in vitro* protects glioma and other malignant cells against apoptosis^{39,40} by phosphorylating and functionally inactivating several pro-apoptotic targets, including the BCL-2 family member BAD and the protease caspase-9.^{41,42} Many malignant glioma cell lines, including D54MG and U87MG cells, constitutively express activated Akt.⁴³ In the context of TRAIL activity, the overexpression of activated Akt can inhibit TRAIL-induced apoptosis.²² However, TRAIL's effect on endogenous Akt remains to be fully elucidated. In this study, TRAIL treatment resulted in a decrease in its phosphorylated form in D54MG, accompanied by cleavage of the protein in TRAIL-sensitive cells. Because the reduction of Akt levels was inhibited by caspase inhibitors, this effect appears to be directly regulated by a caspase-dependent mechanism. Supporting this possibility, we noted the appearance of a 44-kDa band in the immunoblot of TRAIL-treated D54MG cells, which was absent in control cells, and appeared at 3 h in association with reduced levels of 60-kDa phosphorylated Akt, likely representing an Akt cleavage product (Figure 4). Incubation of Akt with activated caspase-3 has previously been reported to result in cleavage of the protein with the formation of 40- and 44-kDa fragments similar to that seen in the present study. In this context, Akt sites for caspase 3-mediated cleavage have been identified at aa108 (TVAD) and aa119 (EEMD), mutation of which results in non-cleavable Akt forms and protects against apoptosis induced by anoikis or Fas.^{18,26} However, in this study, D54MG cells overexpressing caspase-3-resistant Akt mutants (108A/119A) showed the same degree of sensitivity to TRAIL as those overexpressing wt Akt. Additionally, the mutant Akt forms underwent degradation to the same extent as the wild-type forms, suggesting that proteases other than caspase-3 were involved in cleavage. A similar finding was reported by Martin *et al.*, who noted cleavage of caspase-3-resistant Akt mutants and lack of protection from apoptosis during H₂O₂-induced cell death.⁴⁴ Hence, TRAIL may activate other caspases such as caspase-7 that could participate in Akt cleavage. To further delineate the specific role of Akt in TRAIL-induced apoptosis, we used a novel Akt-specific inhibitor, SH-6, that does not affect the activity of other members of this pathway, such as PI3-kinase and PDK1. Increased TRAIL sensitivity was noted after Akt inhibition, providing further support for the protective role of Akt against TRAIL-induced apoptosis. The findings of this study could have relevance to cancer therapy given the continued preclinical progress of treatment approaches utilizing the TRAIL apoptotic pathway and could provide a rationale for combining Akt inhibitors with strategies that activate the TRAIL receptors.

JNK, a key mediator of inflammatory responses and a component of the stress-activated kinase pathway,^{45,46} is activated by phosphorylation in response to various stimuli that are

related to cellular stress, including cytokines, chemotherapy agents, ionizing radiation, and UV exposure.⁴⁷ TNF family members such as Fas and TNF- α can also activate JNK. The significance of such activation in the induction of apoptosis is uncertain, but it appears to depend on the particular pathway that is involved, the duration of activation, as well as cell type.^{20,48–50} TNF activates JNK in a biphasic manner with an early transient activation preceding caspase activity and a later prolonged phase, which is temporally associated with caspase activation and the induction of apoptosis. In contrast, only the prolonged phase of JNK activation is associated with Fas-L and appears to correlate with caspase activity.⁵¹ The activation of JNK that we observed following exposure to TRAIL most closely resembled the kinetics of Fas-induced JNK activation. The temporal profile correlated with the kinetics of caspase activation in D54MG cells. In keeping with this observation, when caspase activity was inhibited by z-DEVD-fmk, JNK activation was abolished. However, when TRAIL-induced JNK activation was inhibited by Forskolin or D-JNK1, the degree of apoptosis remained unchanged, suggesting that JNK activation does not contribute to apoptosis in glioma cells. This finding contrasts with the results of Herr *et al.* that JNK activation contributed to TRAIL-induced apoptosis in lymphoid cells,⁵² but is similar to the finding by Low *et al.* that inhibiting JNK activation associated with Fas activation did not affect the induction of apoptosis.⁵³

TRAIL induces apoptosis in gliomas independent of their p53 status and negates the protective effects of Akt and FLIP, thus promoting apoptosis. Agents targeting TRAIL receptors against malignancies, including anti-DR4 and anti-DR5 antibodies, which have been extensively investigated in preclinical studies, are currently in clinical trials. Our results suggest that TRAIL activates death pathways in glioma cells and also downregulates critical survival factors that might otherwise provide protection for the cells, a finding that has implications for the potential therapeutic use of TRAIL against this malignancy.

Acknowledgments

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Abbreviations

TRAIL	TNF-related apoptosis inducing ligand
TNF	tumor necrosis factor
JNK	c-jun N-terminal kinase
DR4 and DR5	death receptors 4 and 5
XTT	sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate)

References

1. Golstein P. Cell death: TRAIL and its receptors. *Curr Biol.* 1997; 7:R750–R753. [PubMed: 9382834]
2. Schneider P, Thome M, Burns K, et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity.* 1997; 7:831–836. [PubMed: 9430228]
3. Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA, Walczak H. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signaling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *The EMBO J.* 2002; 21:4520–4530.

4. Wajant H, Johannes FJ, Haas E, et al. Dominant-negative FADD inhibits TNFR60-, Fas/Apo1- and TRAIL-R/Apo2-mediated cell death but not gene induction. *Curr Biol*. 1998; 8:113–116. [PubMed: 9427646]
5. Yamada H, Tada-Oikawa S, Uchida A, Kawanishi S. TRAIL Causes Cleavage of Bid by Caspase-8 and Loss of Mitochondrial Membrane Potential Resulting in Apoptosis in BJAB Cells. *Biochem Biophys Res Commun*. 1999; 265:130–133. [PubMed: 10548502]
6. Suliman A, Lam A, Datta R, Srivastava RK. Intracellular mechanisms of TRAIL: Apoptosis through mitochondrial-dependent and- independent pathways. *Oncogene*. 2001; 20:2122–2133. [PubMed: 11360196]
7. Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes Develop*. 2002; 16:33–45. [PubMed: 11782443]
8. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*. 1997; 277:815–818. [PubMed: 9242610]
9. Marsters SA, Sheridan JP, Pitti RM, et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol*. 1997; 7:1003–1006. [PubMed: 9382840]
10. Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol*. 1998; 161:2833–2840. [PubMed: 9743343]
11. Mitsiades N, Poulaki V, Mitsiades C, Tsokos M. Ewing's Sarcoma Family Tumors Are Sensitive to Tumor Necrosis Factor-related Apoptosis-inducing Ligand and Express Death Receptor 4 and Death Receptor 5. *Cancer Res*. 2001; 61:2704–2712. [PubMed: 11289151]
12. Hao C, Beguinot F, Condorelli G, et al. Induction and Intracellular Regulation of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Mediated Apoptosis in Human Malignant Glioma Cells. *Cancer Res*. 2001; 61:1162–1170. [PubMed: 11221847]
13. Marsters SA, Pitti RA, Sheridan JP, Ashkenazi A. Control of apoptosis signaling by Apo2 ligand. *Recent Prog Horm Res*. 1999; 54:225–234. [PubMed: 10548878]
14. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science*. 1997; 277:818–821. [PubMed: 9242611]
15. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest*. 1999; 104:155–162. [PubMed: 10411544]
16. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis- inducing ligand *in vivo*. *Nat Med*. 1999; 5:157–163. [PubMed: 9930862]
17. Kozikowski AP, Sun H, Brognard J, Dennis PA. Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. *J Am Chem Soc*. 2003; 125:1144–1145. [PubMed: 12553797]
18. Rokudai S, Fujita N, Hashimoto Y, Tsuruo T. Cleavage and inactivation of antiapoptotic Akt/PKB by caspases during apoptosis. *J Cell Physiol*. 2000; 182:290–296. [PubMed: 10623893]
19. Scaffidi C, Schmitz I, Krammer PH, Peter ME. The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem*. 1999; 274:1541–1548. [PubMed: 9880531]
20. Chen YR, Wang X, Templeton D, Davis RJ, Tan TH. The Role of c-Jun N-terminal Kinase (JNK) in Apoptosis Induced by Ultraviolet C and gamma Radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem*. 1996; 271:31929–31936. [PubMed: 8943238]
21. Kharbanda S, Ren R, Pandey P, et al. Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature*. 1995; 376:785–788. [PubMed: 7651539]
22. Chen X, Thakkar H, Tyan F, et al. Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer. *Oncogene*. 2001; 20:6073–6083. [PubMed: 11593415]
23. Secchiero P, Gonelli A, Carnevale E, et al. TRAIL Promotes the Survival and Proliferation of Primary Human Vascular Endothelial Cells by Activating the Akt and ERK Pathways. *Circulation*. 2003; 107:2250–2256. [PubMed: 12668516]
24. Kandasamy K, Srivastava RK. Role of the Phosphatidylinositol 3 -Kinase/PTEN/Akt Kinase Pathway in Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in Non-Small Cell Lung Cancer Cells. *Cancer Res*. 2002; 62:4929–4937. [PubMed: 12208743]

25. Zinda MJ, Vlahos CJ, Lai MT. Ceramide induces the dephosphorylation and inhibition of constitutively activated Akt in PTEN negative U87mg cells. *Biochem Biophys Res Commun*. 2001; 280:1107–1115. [PubMed: 11162641]
26. Bachelder RE, Wendt MA, Fujita N, Tsuruo T, Mercurio AM. The Cleavage of Akt/Protein Kinase B by Death Receptor Signaling Is an Important Event in Detachment-induced Apoptosis. *J Biol Chem*. 2001; 276:34702–34707. [PubMed: 11463786]
27. Wilson DJ, Fortner KA, Lynch DH, et al. JNK, but not MAPK, activation is associated with Fas-mediated apoptosis in human T cells. *Eur J Immunol*. 1996; 26:989–994. [PubMed: 8647190]
28. Herr I, Wilhelm D, Bohler T, Angel P, Debatin KM. JNK/SAPK activity is not sufficient for anticancer therapy-induced apoptosis involving CD95-L, TRAIL and TNF-alpha. *Int J Cancer*. 1999; 80:417–424. [PubMed: 9935184]
29. Fukazawa T, Fujiwara T, Morimoto Y, et al. Differential involvement of the CD95 (Fas/APO-1) receptor/ligand system on apoptosis induced by the wild-type p53 gene transfer in human cancer cells. *Oncogene*. 1999; 18:2189–2199. [PubMed: 10327065]
30. Rokhlin OW, Gudkov AV, Kwek S, Glover RA, Gewies AS, Cohen MB. p53 is involved in tumor necrosis factor-alpha-induced apoptosis in the human prostatic carcinoma cell line LNCaP. *Oncogene*. 2000; 19:1959–1968. [PubMed: 10773886]
31. Reles A, Wen WH, Schmider A, et al. Correlation of p53 Mutations with Resistance to Platinum-based Chemotherapy and Shortened Survival in Ovarian Cancer. *Clinical Cancer Research*. 2001; 7:2984–2997. [PubMed: 11595686]
32. Haas-Kogan DA, Kogan SS, Yount G, et al. p53 function influences the effect of fractionated radiotherapy on glioblastoma tumors. *Int J Radiat Oncol Biol Phys*. 1999; 43:399–403. [PubMed: 10030268]
33. Sheikh MS, Burns TF, Huang Y, et al. 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*. 1998; 58:1593–1598. [PubMed: 9563466]
34. Fulci G, Labuhn M, Maier D, et al. p53 gene mutation and ink4a-arf deletion appear to be two mutually exclusive events in human glioblastoma. *Oncogene*. 2000; 19:3816–3822. [PubMed: 10949938]
35. Xiao C, Yang BF, Asadi N, Beguinot F, Hao C. Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Death-inducing Signaling Complex and Its Modulation by c-FLIP and PED/PEA-15 in Glioma Cells. *J Biol Chem*. 2002; 277:25020–25025. [PubMed: 11976344]
36. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW. Phosphatidylinositol 3-Kinase/Akt Activity Regulates c-FLIP Expression in Tumor Cells. *J Biol Chem*. 2001; 276:6893–6896. [PubMed: 11145953]
37. Mitsiades N, Mitsiades CS, Poulaki V, Anderson KC, Treon SP. Intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human multiple myeloma cells. *Blood*. 2002; 99:2162–2171. [PubMed: 11877293]
38. Leverkus M, Neumann M, Mengling T, et al. Regulation of Tumor Necrosis Factor-related Apoptosis-inducing Ligand Sensitivity in Primary and Transformed Human Keratinocytes. *Cancer Res*. 2000; 60:553–559. [PubMed: 10676636]
39. Franke TF, Kaplan DR, Cantley LC. PI3K: Downstream AK-Tion blocks apoptosis. *Cell*. 1997; 88:435–437. [PubMed: 9038334]
40. Bowers DC, Fan S, Walter KA, et al. Scatter Factor/Hepatocyte Growth Factor Protects against Cytotoxic Death in Human Glioblastoma via Phosphatidylinositol 3-Kinase- and AKT-dependent Pathways. *Cancer Res*. 2000; 60:4277–4283. [PubMed: 10945642]
41. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. 1997; 91:231–241. [PubMed: 9346240]
42. Cardone MH, Roy N, Stennicke HR, et al. Regulation of Cell Death Protease Caspase-9 by Phosphorylation. *Science*. 1998; 282:1318–1321. [PubMed: 9812896]
43. Schlegel J, Piontek G, Budde B, Neff F, Kraus A. The Akt/protein kinase B-dependent anti-apoptotic pathway and the mitogen-activated protein kinase cascade are alternatively activated in human glioblastoma multiforme. *Cancer Lett*. 2000; 158:103–108. [PubMed: 10940516]

44. Martin D, Salinas M, Fujita N, Tsuruo T, Cuadrado A. Ceramide and Reactive Oxygen Species Generated by H₂O₂ Induce Caspase-3-independent Degradation of Akt/Protein Kinase B. *J Biol Chem*. 2002; 277:42943–42952. [PubMed: 12213802]
45. Sluss HK, Barrett T, Derijard B, Davis RJ. Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol Cell Biol*. 1994; 14:8376–8384. [PubMed: 7969172]
46. Cahill MA, Peter ME, Kischkel FC, et al. CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. *Oncogene*. 1996; 13:2087–2096. [PubMed: 8950975]
47. Adler V, Fuchs SY, Kim J, et al. jun-NH2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells. *Cell Growth Differ*. 1995; 6:1437–1446. [PubMed: 8562482]
48. Verheij M, Bose R, Lin XH, et al. Requirement for ceramide-initiated SAPK/JNK signalling in stress- induced apoptosis. *Nature*. 1996; 380:75–79. [PubMed: 8598911]
49. Eilers A, Whitfield J, Babij C, Rubin LL, Ham J. Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. *J Neurosci*. 1998; 18:1713–1724. [PubMed: 9464996]
50. Zanke BW, Boudreau K, Rubie E, et al. The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr Biol*. 1996; 6:606–613. [PubMed: 8805279]
51. Roulston A, Reinhard C, Amiri P, Williams LT. Early Activation of c-Jun N-terminal Kinase and p38 Kinase Regulate Cell Survival in Response to Tumor Necrosis Factor alpha. *J Biol Chem*. 1998; 273:10232–10239. [PubMed: 9553074]
52. Herr I, Wilhelm D, Meyer E, Jeremias I, Angel P, Debatin KM. JNK/SAPK activity contributes to TRAIL-induced apoptosis. *Cell Death Differ*. 1999; 6:130–135. [PubMed: 10200559]
53. Low W, Smith A, Ashworth A, Collins M. JNK activation is not required for Fas-mediated apoptosis. *Oncogene*. 1999; 18:3737–3741. [PubMed: 10391681]

Figure 1.

TRAIL activates caspase8 and induces apoptosis in glioma cells. (A) Flowcytometric analysis of TRAIL-treated cells showing induction of apoptosis as a sub-G1 fraction. Cells were harvested after treatment with TRAIL for 24 h, fixed in ethanol, and stained with propidium iodide prior to analysis by flow cytometry. (B) Caspase-8 activation was determined by immunoblot analysis in D54MG cells treated with TRAIL (1 μ g/ml). The cells were pretreated with the caspase-8 inhibitor, z-IETD-fmk (50 μ M), or the caspase-3 inhibitor, z-DEVD-fmk (50 μ M) for 4 h prior to treatment with TRAIL. Caspase-8 levels were assessed by immunoblot analysis.

Figure 2.

TRAIL activates the mitochondrial apoptotic pathway. (A) D54MG and U87MG cells were harvested after treatment with TRAIL (1 μ g/ml) for the time periods indicated. BID cleavage was analyzed by Western blot analysis. To assess cyt C release from the mitochondria, cells were harvested, lysed, and centrifuged to separate the cytoplasmic fraction from the subcellular organelle and nuclear fractions. The cytoplasmic fraction was analyzed for the presence of cyt C, indicating its release from the mitochondria. (B) Cells were pretreated with either a caspase-8 (z-IETD-fmk) or caspase-3 (z-DEVD-fmk) inhibitor for 4 h prior to adding TRAIL. BID levels and cyt C release were assessed by Western blot analysis.

Figure 3.

Levels of FLIP are downregulated in response to TRAIL. Glioma cells were treated with TRAIL and the effect on FLIP levels was assessed by immunoblot analysis. FLIP levels decreased in cells that were sensitive to TRAIL-induced apoptosis. Note the 44 kd cleavage product in D54 cells.

Figure 4. TRAIL downregulates endogenous Akt levels. (A) D54MG and U87MG cells were exposed to TRAIL for the time periods indicated and the levels of phosphorylated (Ser-473 and Thr-308) and total Akt were determined by immunoblotting. (B) Cells were pretreated with caspase inhibitors, z-IETD-fmk and z-DEVD-fmk, and exposed to TRAIL. Total Akt expression was determined by immunoblotting with Actin as a loading control.

Figure 5.

Glioma cells overexpressing Akt mutants resistant to caspase-3 cleavage remain sensitive to TRAIL. (a) D54MG cells were transiently transfected with either vector alone or vectors overexpressing wt Akt or Akt mutants (108A, 119A or 108A/119A). The cells were exposed to TRAIL 24 h after transfection and the levels of total Akt were determined by immunoblot analysis. An anti-FLAG (M2) antibody was used to assess the levels of expression of the FLAG-tagged mutant proteins. (b) Following transfection with control vector or Akt mutant constructs, the cells were exposed to TRAIL and degree of apoptosis (sub-G1 fraction) was assessed by flowcytometric analysis.

Figure 6.

Inhibition of Akt sensitizes glioma cells to TRAIL. D54MG cells were treated with the Akt-specific inhibitor SH-6 (20 μ M) for 24 h and then exposed to TRAIL for an additional 24 h. (a) Levels of expression of phosphorylated Akt, total Akt, FLIP and Actin were determined by immunoblot analysis. (b) Cells were harvested, fixed with ethanol and stained with propidium iodide for determining the degree of apoptosis by flowcytometry.

Figure 7.

TRAIL treatment results in JNK activation of JNK in glioma cells. A D54MG cells were treated with $1\mu\text{g/ml}$ TRAIL for the duration indicated and JNK activity was immunoprecipitated from total protein using anti-JNK antibodies. A kinase reaction was set up with the precipitates by adding GST-jun-(1–331), ATP, and $[^{32}\text{P}]\text{ATP}$ for 20 min at 30°C . The reaction products were resolved by SDS-PAGE and autoradiography. Total JNK levels assessed by immunoblotting were used to normalize the relative kinase activities. For caspase inhibition, the cells were pretreated with z-IETD-fmk or z-DEVD-fmk for 4 h prior to treatment with TRAIL.

Figure 8.

JNK activation by TRAIL is independent of apoptosis induction. (a) D54MG cells were treated with the JNK inhibitor, TAT-JNKI with appropriate controls and then exposed to TRAIL. JNK activation was assessed by a kinase assay and immunoblotting to determine the level of c-jun phosphorylation. (b) D54MG cells were pretreated with TAT-JNKI and subsequently exposed to TRAIL. The percentage of apoptotic cells was determined as the sub-G1 fraction by flow cytometry. (c) JNK activation is unrelated to concentration of TRAIL (upper panel); the degree of apoptosis remained independent of JNK activation at lower concentrations of TRAIL (lower panel).