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Intracellular Regulation of TRAIL-Induced Apoptosis in Human Melanoma Cells

Thomas S. Griffith,[1] Wilson A. Chin, Glenn C. Jackson, David H. Lynch, and Marek Z. Kubin

The observation that TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF cytokine family, induces apoptosis in a number of different tumor cell types led us to compare the tumoricidal effects of TRAIL to those of other TNF family molecules on human melanoma cells. We found that a high proportion of the melanoma cell lines tested were killed by TRAIL, whereas all the melanoma lines were resistant to the other TNF family cytokines tested. TRAIL-induced death was characterized by caspase activation and cellular protein cleavage within minutes of TRAIL addition, and death could be completely inhibited by the caspase inhibitors Ile-Glu-Thr-Asp (IETD) and Val-Ala-Asp (VAD), indicating the presence of a TRAIL receptor signaling pathway similar to that identified for Fas and TNF receptors. Specific TRAIL receptor expression was determined by RT-PCR, and the presence of mRNA encoding the "protective" TRAIL receptors did not correspond to resistance or sensitivity to TRAIL-induced apoptosis. Addition of protein synthesis inhibitors to TRAIL-resistant melanomas rendered them sensitive to TRAIL, indicating that the presence or the absence of intracellular apoptosis inhibitors may mediate resistance or sensitivity to TRAIL-mediated apoptosis. Expression of one such inhibitor, FLICE-inhibitory protein (FLIP), was highest in the TRAIL-resistant melanomas, while being low or undetectable in the TRAIL-sensitive melanomas. Furthermore, addition of actinomycin D to TRAIL-resistant melanomas resulted in decreased intracellular concentrations of FLIP, which correlated with their acquisition of TRAIL sensitivity. Collectively, our results indicate that TRAIL-induced apoptosis occurs through a caspase signaling cascade and that resistance is controlled by intracellular regulators of apoptosis. ***The Journal of Immunology*****, 1998, 161: 2833–2840.**

Programmed cell death, or apoptosis, is a vital process in the life of complex organisms, and this death is regulated in situ by many intracellular and extracellular signals. One growing group of molecules that function as inhibitors and activators of apoptosis is the TNF family of cytokines and receptors (1). For example, CD40 ligand (CD40L)[2] has been shown to inhibit apoptotic cell death (2–4), whereas TNF and Fas ligand (FasL) have been shown to function as inducers of apoptosis in many physiologic events, such as autoimmunity, activation-induced cell death, immune privilege, and evasion of tumors from the immune system (5–10).

TRAIL (TNF-related apoptosis-inducing ligand) is a recently identified member of the TNF family that, like FasL, is a type II membrane protein capable of inducing apoptotic cell death in a variety of cell types (11, 12). TRAIL can interact with four distinct receptors: DR4 (13), DR5/TRAIL-R2/TRICK2 (14–17), TRID/DcR1/TRAIL-R3/LIT (14, 15, 18, 19), and TRAIL-R4/DcR2 (20, 21) (hereafter referred to as TRAIL-R1, -R2, -R3, and -R4, respectively). Both TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins that contain cytoplasmic death domains and, upon ligation, mediate apoptosis (13–16). In contrast, neither TRAIL-R3 (which is glycosylphosphatidylinositol (GPI) linked) nor TRAIL R-4 (which is a type I transmembrane protein that contains an incomplete cytoplasmic death domain) mediates apoptosis upon ligation with TRAIL (14, 15, 18, 20, 21). Because they lack the ability to directly signal cell death, TRAIL-R3 and TRAIL-R4 have been hypothesized as being protective receptors, either by acting as "decoy" receptors (14, 15, 21) or via transduction of an antiapoptotic signal (20).

The mRNA distribution of TRAIL, TRAIL-R1, and TRAIL-R2 is broad, with many of the same tissues expressing transcripts for both TRAIL and these TRAIL receptors, whereas the distribution of TRAIL-R3 and -R4 is more restricted (11, 13, 16, 18, 20). Although the in vivo role of the TRAIL/TRAIL receptor system is not currently known, in vitro studies have found normal tissues to be resistant to TRAIL-induced death, and some tumor cell lines to be sensitive to the cytotoxic effects of TRAIL (11, 12). While it has been suggested that the presence or the absence of the nonsignaling receptors may determine whether a cell is resistant or sensitive, respectively, to TRAIL-induced apoptosis, it seems unlikely that this would be the only mechanism controlling survival upon TRAIL binding. Regardless of the mechanism of selectivity, these early reports still implicate TRAIL as a potential tumor therapeutic, where tumor cells would be induced to undergo apoptosis and cells in normal tissues would not. In the current study we compared the effectiveness of TRAIL to that of a panel of TNF family cytokines for their ability to induce death in a number of human melanoma cell lines. Our results provide information concerning the activation and regulation of TRAIL-induced apoptosis, and suggest a potential use for TRAIL as a treatment for human melanoma.

Materials and Methods

Materials

The tetrapeptide caspase inhibitors, carbobenzyloxy-Val-Ala-Asp (OMe) fluoromethyl ketone (z-VAD-fmk), carbobenzyloxy-Asp-Glu-Val-Asp fluoromethyl ketone (z-DEVD-fmk), and carbobenzyloxy-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (z-IETD-fmk), were obtained from Enzyme Systems Products (Livermore, CA). Stock solutions of the inhibitors

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[2] Abbreviations used in this paper: CD40L, CD40 ligand; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase; FLIP, FLICE-inhibitory protein; PBSA, 3% BSA in PBS; RGP, radial growth phase; VGP, vertical growth phase.

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were prepared in DMSO and stored at 4°C. The Abs against caspase-8 (provided by Dr. M. Peter, Heidelberg, Germany), caspase-3 (Transduction Laboratories, Lexington, KY), and poly(ADP-ribose) polymerase (PARP; PharMingen, San Diego, CA) were used according to the manufacturer's instruction. Antiserum against human FLICE-inhibitory protein (FLIP) was generated by injecting rats with a peptide spanning amino acids 2 to 26 (SAEVIHQVEEALDTDEKEMLFLCRD) (22). The FLIP peptide was synthesized on solid support resins (Novabiochem, La Jolla, CA) on an Applied Biosystems 433A peptide synthesizer (Foster City, CA) using F-moc chemistry (23). Peptides were cleaved from the resin using a cleavage mixture (1/2/2/3/40, ethanedithiol/thioanisole/water/phenol/trifluoroacetic acid) and were purified on a Vydac C_{18} column (Resolution Systems, Wilmette, IL) using a 0 to 60% acetonitrile gradient in 0.1% trifluoroacetic acid. The identity and purity of the peptide were confirmed by HPLC, amino acid analysis, and mass spectrometry using a PerSeptive Biosystems Voyager-DE STR Biospectrometer (Framingham, MA).

Cell lines

Human melanoma cell lines were provided by Dr. M. Herlyn (WM 9, 35, 98-1, 164, 793, 1205-Ln, 1791-C, and 3211; Wistar Institute, Philadelphia, PA) and were cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, and glutamine. Normal human epidermal melanocytes were obtained from Clonetics (San Diego, CA) and cultured as directed.

In vitro killing of human cell lines with TNF family molecules

Tumor sensitivity to TRAIL, CD40L, TNF-α, and FasL was assayed by incubating the cells in 96-well plates (5×10^4 cells/well) with purified LZ-TRAIL (300 ng/ml) (16), LZ-CD40L (20 μg/ml), TNF-α (300 ng/ml), or culture supernatant containing LZ-FasL (1 μg/ml, as determined by Western blot) for 24 h. The indicated values were starting concentrations, followed by threefold dilutions. In some experiments, actinomycin D (10 ng/ml) or cycloheximide (10 μM) was added to the culture medium immediately before the addition of LZ-TRAIL. Cell death was determined by crystal violet staining as previously described (24). Results are presented as the percent cell death: $1 -$ (OD of cells treated with LZ-TRAIL per OD of cells not treated with LZ-TRAIL) $\times$ 100. Cell lines were considered sensitive if there was >30% cell death induced by the highest concentration of LZ-TRAIL, LZ-CD40L, TNF-α, or LZ-FasL.

Flow cytometry

Surface expressions of TRAIL receptor(s), CD40, and Fas were determined by flow cytometric analysis by measuring the binding of LZ-TRAIL, LZ-CD40L, and anti-Fas Ab (M3). Briefly, cells were incubated with 10 μg/ml LZ-TRAIL, LZ-CD40L, or M3 in 3% BSA in PBS (PBSA) for 30 min on ice. Following three washes with PBS, cells were incubated with a mouse anti-leucine zipper Ab (M15; 10 μg/ml in 3% PBSA) for 30 min on ice. Finally, after three washes in PBS, the cells were incubated for 30 min on ice with a goat anti-mouse FITC-conjugated Ab (diluted 1/200 in 3% PBSA; Sigma, St. Louis, MO). Cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA).

Western blot analysis

Cells from each melanoma line were lysed in PBS containing 1% Nonidet P-40, 0.35 mg/ml PMSF, 9.5 μg/ml leupeptin, and 13.7 μg/ml pepstatin A. The lysed cells were centrifuged at 14,000 $\times$ *g* to remove cellular debris. Protein concentrations of the extracts were determined by the colorimetric bicinchoninic acid analysis (Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane (Novex, San Diego, CA), and blocked with 5% nonfat dry milk in PBS-Tween-20 (0.05%, v/v) overnight. The membrane was incubated with the anti-caspase-8, anti-caspase-3, or anti-PARP Abs (diluted according to the manufacturer's instructions) or with FLIP antiserum (diluted 1/1000) for 1 h. After washing, the membrane was incubated with an anti-mouse horseradish peroxidase or anti-rat horseradish peroxidase Ab (diluted 1/1000; Amersham, Arlington Heights, IL) for 1 h. Following several washes, the blots were developed by chemiluminescence according to the manufacturer's protocol (Renaissance chemiluminescence reagent, DuPont-New England Nuclear, Boston, MA).

RT-PCR for human TRAIL receptors

Total RNA was isolated from various human cell lines with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA samples (1 μg each) were tested for DNA contamination by 30 cycles of PCR with human β-actin primers. After it was shown that there was no DNA contamination, cDNA synthesis was performed using an RNA PCR kit (Perkin-Elmer, Norwalk, CT) with the supplied oligo$(dT)_{16}$ primer. RT was performed using a thermal program of 25°C for 10 min, 42°C for 30 min, and 95°C for 5 min. PCR reactions were performed using the following primers: β-actin (forward: 5′-GAAACTACCTTCAACTCCATC-3′; reverse: 5′-CGAGGCCAGGATGGAGCCGCC-3′), TRAIL-R1 (forward: 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′; reverse: 5′-TCCAAGGACACGGCAGAGCCTGTGCCAT-3′), TRAIL-R2 (forward: 5′-GCCTCATGGACAATGAGATAAAGGTGGCT-3′; reverse: 5′-CCAAATCTCAAAGTACGCACAAACGG-3′), TRAIL-R3 (forward: 5′-GAAGAATTTGGTGCCAATGCCACTG-3′; reverse: 5′-CTCTTGGACTTGGCTGGGAGATGTG-3′), and TRAIL-R4 (forward: 5′-CTTTTCCGGCGGCGTTCATGTCCTTC-3′; reverse: 5′-GTTTCTTCCAGGCTGCTTCCCTTTGTAG-3′), giving products of 219, 506, 502, 612, and 453 bp, respectively. Human β-actin PCR cycle conditions were 95°C for 45 s, 55°C for 1 min, and 72°C for 45 s for 30 cycles. Human TR-1, -2, and -3 conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. Human TR-4 cycle conditions were 95°C for 4 min 15 s, followed by 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide.

Results

Sensitivity of human melanoma cell lines to TRAIL

Based on the fact that members of the TNF family are cytotoxic to a number of human tumor cell lines (25), we were interested in comparing the effectiveness of TRAIL to induce death in human melanoma cell lines and normal melanocytes to that of three other TNF family molecules (CD40L, FasL, and TNF). Five of the lines (WM 9, WM 35, WM 98-1, WM 793, and WM 1205 Ln) were sensitive to the cytotoxic effects of TRAIL, whereas three lines (WM 164, WM 1791-C, and WM 3211) and normal human melanocytes were resistant (Fig. 1). In contrast, all the cell lines were resistant to CD40L, FasL, and TNF.

The possibility that resistance of the melanoma cell lines to the various TNF family molecules was due to failure of the cells to express receptors for the various ligands was addressed by flow cytometry. The data demonstrate binding of TRAIL- and Fas-specific mAb to all the cell lines tested (Fig. 2). CD40L binding was also detected with most of the cell lines, but much lower binding was seen with WM 98-1 and WM 164 cells, which may explain why these cell lines were resistant to CD40L-induced apoptosis. Thus, resistance of melanoma cells to cytokine-induced apoptosis is not due to the lack of cognate receptors, but instead appears to be due to other regulatory mechanisms.

Role of caspases in TRAIL-induced death

Apoptotic cell death induced by TNF or FasL is a rapid biochemical process characterized by the activation of a cascade of intracellular proteases, or caspases, and cleavage of numerous intracellular proteins (26, 27). It has been recently suggested that many of the molecules involved in other death receptor-mediated apoptosis also participate in TRAIL-induced death (28, 29). Thus, we wanted to determine the kinetics of caspase activation and cellular protein cleavage during TRAIL-induced apoptosis. The TRAIL-sensitive melanoma WM 1205 Ln was exposed to TRAIL for various periods of time, after which the cells were lysed, and the cellular proteins were separated by SDS-PAGE for Western blot analysis of caspase-8 (FLICE) and caspase-3 (CPP32) activation and PARP cleavage. Caspase-8 activation was detected within 5 min after TRAIL addition to WM 1205 Ln (Fig. 3*A*). Similarly, activation of caspase-3 was detected within 15 min, and PARP cleavage was detected within 30 min after the addition of TRAIL. These results demonstrate the rapid activation of a caspase cascade similar to that seen with other death receptors, leading to the cleavage of intracellular proteins within TRAIL-sensitive cells. Since one of the early biochemical events in apoptotic cell death is the cleavage of PARP from its native 116-kDa form to an 85-kDa fragment

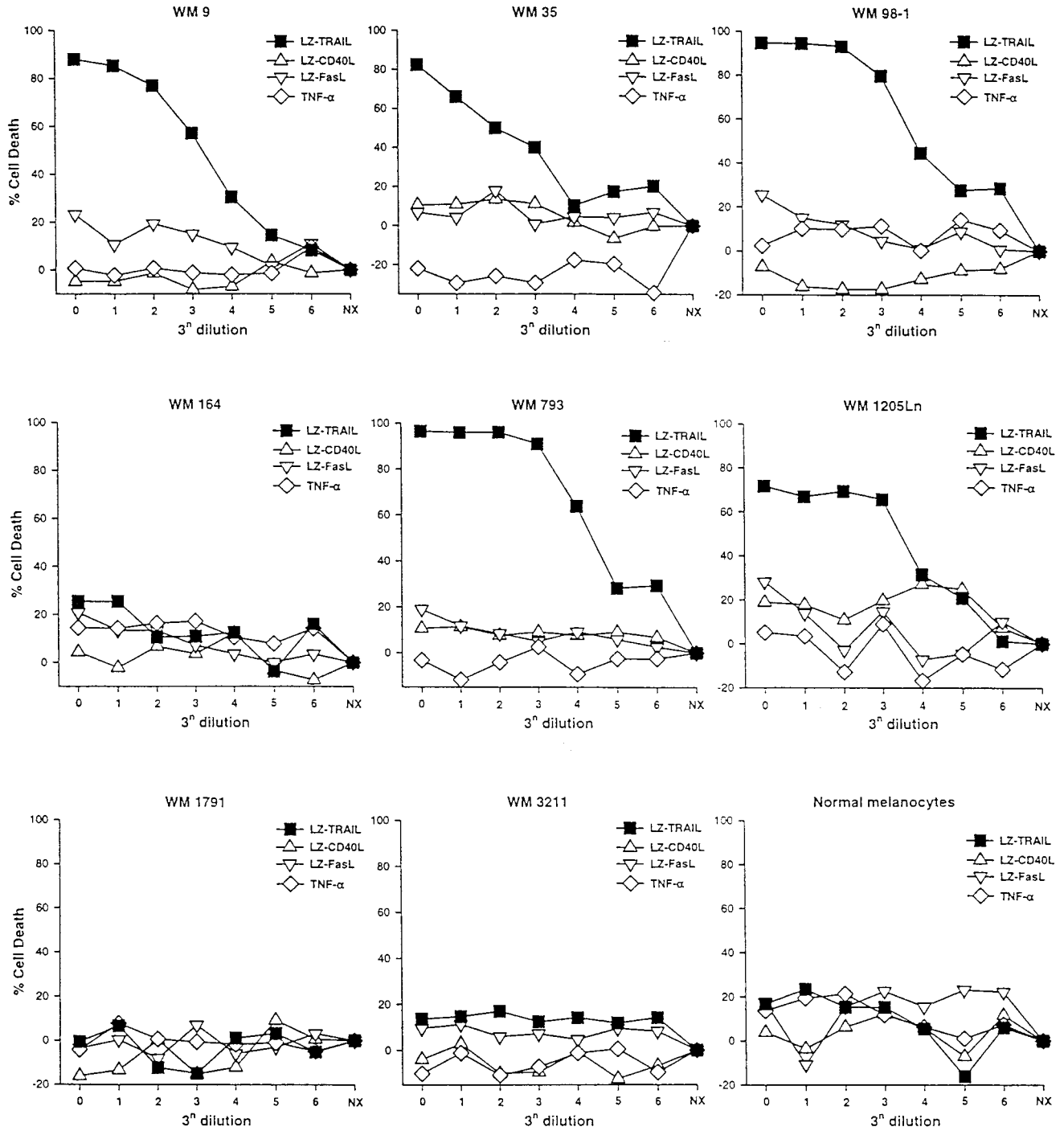

FIGURE 1. Cytotoxicity of TNF family molecules to human melanoma cells and normal human melanocytes. Ninety-six-well microtiter plates were seeded with 5×10^4 cells/well and allowed to adhere for at least 6 h before adding LZ-TRAIL (starting concentration, 300 ng/ml), LZ-CD40L (starting concentration, 20 μg/ml), LZ-FasL (starting concentration, 1 μg/ml), or TNF-α (starting concentration, 300 ng/ml). Cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph, but were <5% for all data points. Experiments were performed at least three separate times with each cell line.

(30), we were interested to determine whether PARP cleavage occurred in all the TRAIL-sensitive melanoma cell lines while it remained uncleaved in the TRAIL-resistant melanomas. Indeed, PARP cleavage was only seen in the TRAIL-sensitive lines and not in the resistant lines (Fig. 3*B*).

To further demonstrate the importance of caspase activation in TRAIL-induced apoptosis, cytotoxicity assays were performed in which the caspase inhibitors z-IETD-fmk, z-DEVD-fmk, and z-VAD-fmk were added to melanoma cells along with TRAIL. In each of the TRAIL-sensitive lines tested, IETD and VAD completely protected the cells from the cytotoxic effects of TRAIL (Fig. 4). In contrast, DEVD was only able to partially inhibit the cytotoxic effects of TRAIL. Equal concentrations of the peptide vehicle (DMSO) did not inhibit TRAIL-induced death. The decreased ability of DEVD to inhibit death compared with that of IETD and VAD may result from differences in the ability of the

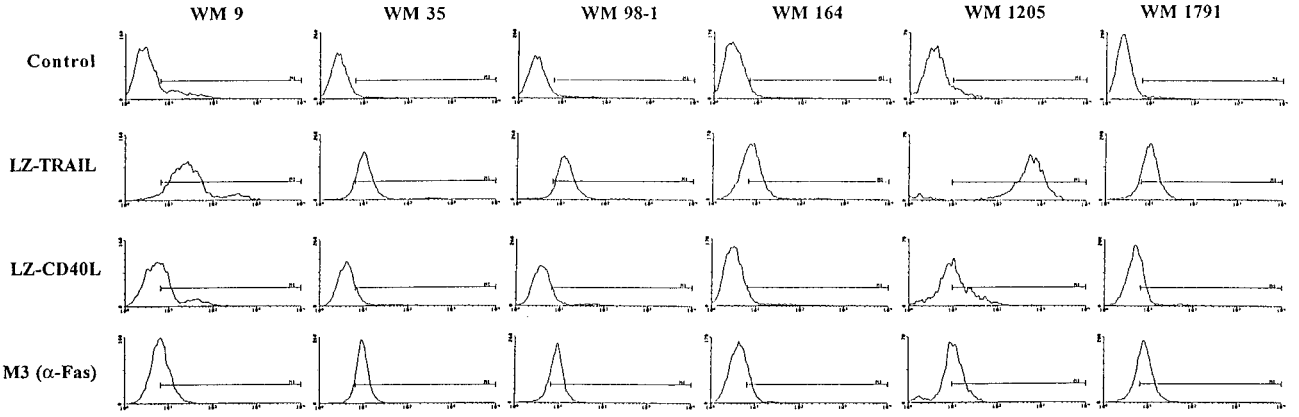

FIGURE 2. Expression of TNF family receptors on human melanoma cells. One million cells were stained with LZ-TRAIL, LZ-CD40L, or M3 (anti-Fas); mouse anti-leucine zipper Ab M15; and FITC goat anti-mouse Ab as described in *Materials and Methods* and were analyzed by flow cytometry.

peptides to enter the cell. These results demonstrate that TRAIL-induced apoptosis shares many of the same characteristics as apoptosis induced by other TNF family molecules.

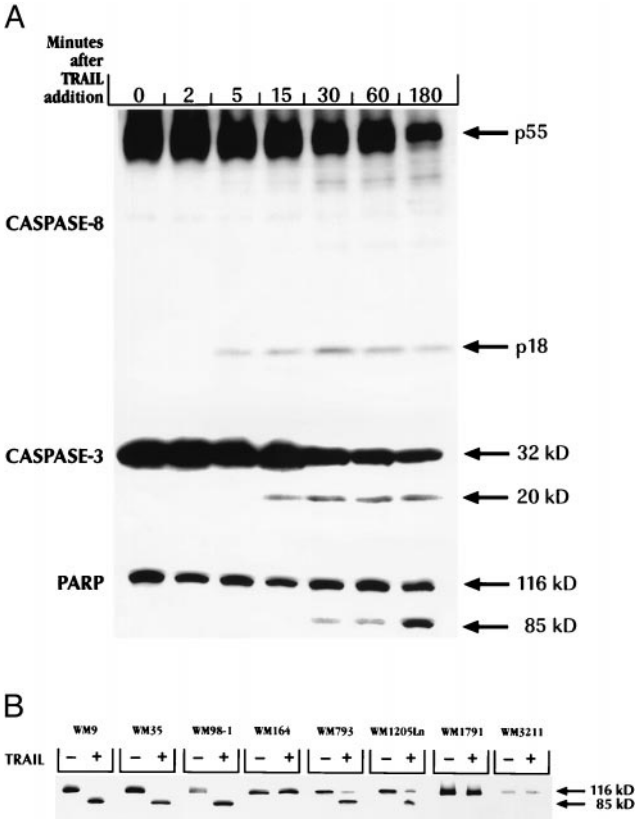

FIGURE 3. Caspase activation and PARP cleavage in TRAIL-induced apoptosis. *A*, Kinetics of caspase-8, caspase-3, and PARP cleavage in the WM 1205 Ln melanoma cell line. Flasks containing 5×10^6 cells were treated with 100 ng/ml LZ-TRAIL for the indicated times, after which cell lysates were prepared, and caspase-8, caspase-3, and PARP cleavage was determined by Western blot analysis. Caspase-8 activation yields an 18-kDa active subunit from the 55-kDa inactive form, while a 20-kDa active subunit is formed from the 32-kDa inactive form during caspase-3 activation. Cleavage of PARP from 116 to 85 kDa occurs during apoptotic cell death. *B*, Sensitivity of melanoma cell lines to TRAIL correlates with PARP cleavage. Flasks containing 5×10^6 cells were treated with 100 ng/ml LZ-TRAIL for 8 h. Cell lysates were prepared, and PARP cleavage was determined by Western blot analysis.

TRAIL receptor expression in human melanoma cell lines as analyzed by RT-PCR

The demonstration that both sensitive and resistant cell lines bind LZ-TRAIL indicated the expression of at least one of the TRAIL receptors on the surface of these tumor cells. Unfortunately, no receptor-specific Abs are available at this time to individually examine the surface expression of these proteins. Thus, oligonucleotide primers derived from unique regions in each receptor sequence were used in RT-PCR to determine which TRAIL receptor transcripts were present in the melanoma cells. Figure 5*A* shows these primer pairs are specific, in that PCR products only resulted when the proper cDNA template was present, and the results from melanoma lines are shown in Figure 5*B*. All the melanomas were positive for TRAIL-R2 mRNA, whereas four of eight were positive for TRAIL-R1, five of eight were positive for TRAIL-R3, and only one was positive for TRAIL-R4. Although there were melanomas (WM 164 and WM 1791) in which TRAIL-R3 and/or -R4 expression correlated with resistance to TRAIL-inducing killing, several of the TRAIL-sensitive lines (WM 9, WM 793, and WM 1205 Ln) were also positive for TRAIL-R3 and/or -R4 mRNA. Alternatively, WM 3211, which is resistant to the cytotoxic effects of TRAIL, tested negative for TRAIL-R3 and -R4 mRNA. Thus, our results indicate that there is no strict correlation between the expression of mRNA encoding TRAIL-R3 or -R4 and sensitivity or resistance to TRAIL-mediated apoptosis.

Protein synthesis inhibitors increase the sensitivity to TRAIL and lead to caspase activation

It is known that the inhibition of protein synthesis can increase the level of sensitivity of target cells that are otherwise resistant to FasL- or TNF-induced death (31). To test the possibility that TRAIL-induced death might also be enhanced by protein synthesis inhibitors, TRAIL-resistant melanoma lines were pretreated with either actinomycin D or cycloheximide, followed by LZ-TRAIL. The melanoma cell line WM 164 is resistant to the cytotoxic effects of TRAIL, but is readily killed upon the addition of either agent to the assay (Fig. 6*A*). Similar results were observed with WM 1791 and WM 3211. Examination of these cells after actinomycin D treatment by RT-PCR found no alteration in the pattern of TRAIL receptor mRNA expression (data not shown). Furthermore, conversion of this cell from resistant to sensitive by actinomycin D correlated with caspase-8 activation and PARP cleavage as detected by Western blot analysis (Fig. 6*B*). These results not only indicate that the "death machinery" is intact and functional within the TRAIL-resistant melanoma lines, but indicate that its

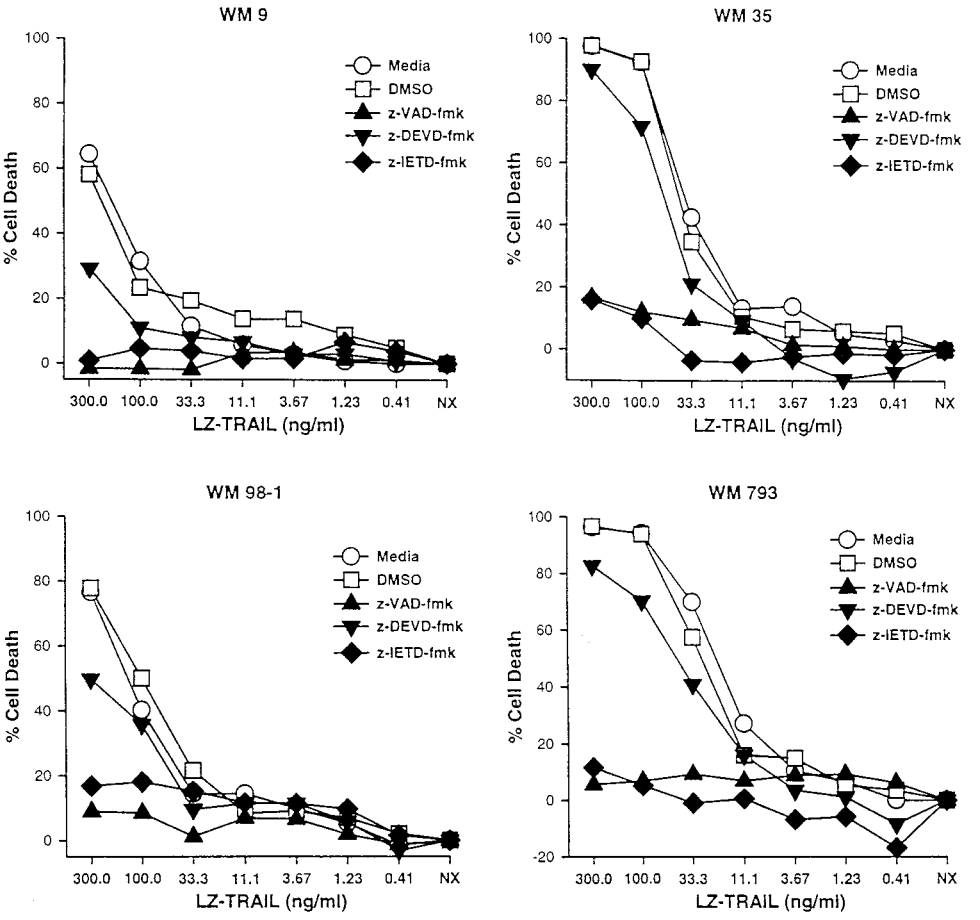

FIGURE 4. Inhibition of TRAIL-induced apoptosis by caspase-inhibiting peptides. Ninety-six-well microtiter plates were seeded with 5×10^4 cells/well and allowed to adhere for at least 6 h. z-IETD-fmk (20 μM), z-DEVD-fmk (20 μM), z-VAD-fmk (20 μM), DMSO, or medium was added to each well immediately before adding LZ-TRAIL. Cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph, but were <5% for all data points. Experiments were performed at least three separate times with each cell line.

function is being inhibited by an intracellular inhibitor(s) of the cell death signaling pathway.

Participation of the apoptosis inhibitory protein FLIP in the resistance of melanomas to TRAIL

The fact that protein synthesis inhibitors can render resistant melanoma cells sensitive to TRAIL suggests that these cells are synthesizing an intracellular inhibitor(s) of apoptosis. Although Bcl-2 and Bcl-x_L have been shown to protect cells from apoptosis induced by FasL and TNF (32–35), only FLIP has been shown to inhibit TRAIL-induced death (22). Thus, FLIP levels in the eight melanoma cell lines used in this study were examined by Western blot with an anti-FLIP antiserum. The TRAIL-resistant melanomas (WM 164, WM 1791, and WM 3211) all expressed high levels of FLIP, whereas FLIP levels in the TRAIL-sensitive melanomas (WM 9, WM 35, WM 98-1, WM 793, and WM 1205 Ln) were low or undetectable (Fig. 7*A*). Caspase-8 levels were also examined in the eight cell lines and were roughly equivalent. The detection of low levels of FLIP in the TRAIL-sensitive lines (WM 9, WM 35, and WM 98-1) suggests that the intracellular concentration of FLIP with respect to that of caspase-8 may determine whether a cell is susceptible to TRAIL.

Our hypothesis that intracellular regulators of apoptosis, namely FLIP, and not the expression of the nonsignaling TRAIL-R3 or -R4 as previously proposed (14, 21), play a vital role in determining whether a cell is sensitive to TRAIL received additional support from the results in Figure 7*B*. If FLIP has a high turnover rate within the cell, then the intracellular levels should rapidly decrease over time when the cells are exposed to actinomycin D. The $FLIP^+$, TRAIL-resistant melanoma WM 3211 was incubated with actinomycin D for various lengths of time, after which the cells were lysed, and caspase-8 and FLIP protein levels were determined by Western blotting. Whereas caspase-8 levels did not dramatically change with actinomycin D treatment, FLIP levels were found to decrease within 1 to 2 h of adding actinomycin D and were significantly lower after 24 h. These results provide additional support for the premise that protection from TRAIL-induced death in melanoma cells is regulated by the ratio of FLIP protein within the cell to the components of the TRAIL receptor death pathway, presumably caspase-8.

Seeing that changes in FLIP levels could be detected within 1 to 2 h of actinomycin D addition, we were interested to see how these changes correlated with the gain in susceptibility to TRAIL-induced death. WM 3211 cells were incubated with actinomycin D for 2, 4, or 6 h, followed by a wash and addition of fresh culture medium. TRAIL was then added, and cell death was determined as previously described. In correlation with the results shown in Figure 7*B*, the sensitivity of WM 3211 to TRAIL increased with the length of actinomycin D pretreatment (Fig. 8). TRAIL-induced death was significantly increased after actinomycin D pretreatment for just 2 h, while pretreating for 6 h increased death levels close to those seen in the control group (actinomycin D and TRAIL for 24 h). Thus, the results indicate that TRAIL-induced death in melanoma cell lines is primarily regulated by the intracellular concentration of FLIP, and not simply by the expression of decoy receptors.

Discussion

The TNF family of molecules is a growing group of cytokines that exert a variety of effects on different cells (36). Perhaps the best

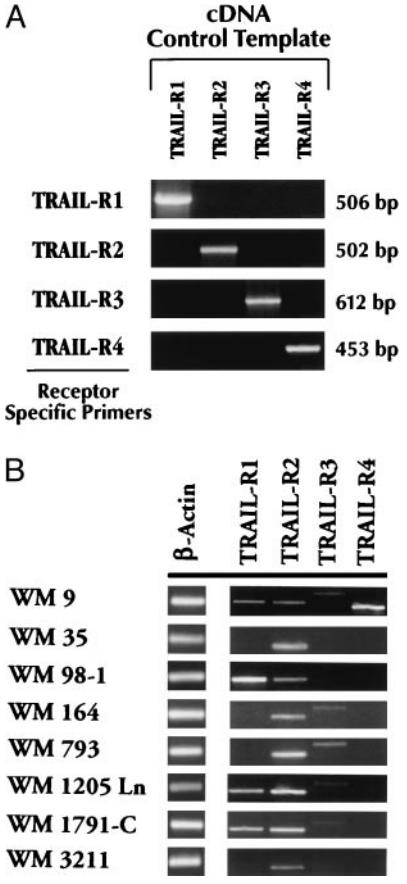

FIGURE 5. Specificity of RT-PCR primer pairs and RT-PCR analysis of TRAIL receptor expression in human tumor cell lines. *A*, RT-PCR primer specificity was confirmed with cDNA templates (50 ng/reaction) of each of the four TRAIL receptors. PCR product sizes for each primer pair are listed. *B*, TRAIL receptor mRNA expression in human tumor cells was determined by RT-PCR as described in *Materials and Methods*.

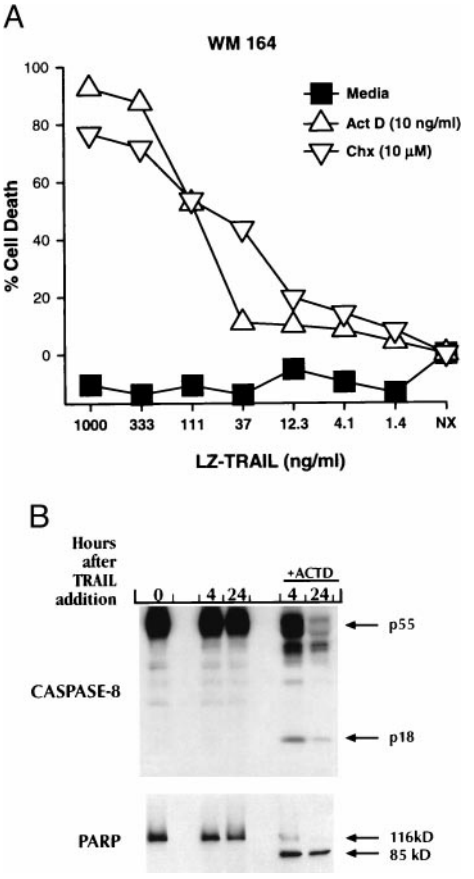

FIGURE 6. Protein synthesis inhibitors make resistant melanomas sensitive to TRAIL and activate caspases. *A*, Ninety-six-well microtiter plates were seeded with WM 164 (5×10^4 cells/well) and allowed to adhere for at least 6 h. Actinomycin D (10 ng/ml) or cycloheximide (10 μM) were added to each well immediately before adding LZ-TRAIL. Cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph, but were <5% for all data points. Experiments were performed at least three separate times with each cell line. *B*, Flasks containing 5×10^6 cells were treated with 100 ng/ml LZ-TRAIL for the indicated times with or without actinomycin D (10 ng/ml), after which cell lysates were prepared, and caspase-8 and PARP cleavage was determined by Western blot analysis. Caspase-8 activation yields an 18-kDa active subunit from the 55-kDa inactive form, while cleavage of PARP from 116 to 85 kDa occurs during apoptotic cell death.

known function of these molecules is the induction of cell death upon binding to their cognate receptors. Although TNF and FasL are extremely efficient at killing a variety of tumor cells, they also cause significant damage to normal tissues that result in life-threatening toxicities (37–39). In this study the effectiveness of TRAIL in killing human melanoma cells exceeded that of other TNF family members. Moreover, TRAIL had the unique characteristic of being cytotoxic only to the tumor cells, with five of eight human melanoma cell lines tested being sensitive to TRAIL-induced apoptosis, while normal melanocytes were unaffected. The finding that TRAIL was cytotoxic to the melanoma cell lines and not to normal melanocytes supports earlier observations (11, 12). Moreover, we have also examined 10 other normal human primary cells and found them all to be resistant to TRAIL-induced death (data not shown).

The identification of four distinct receptors for TRAIL, two with death-inducing ability and two without, was initially proposed as a mechanism by which sensitivity to TRAIL-induced apoptosis was regulated, since the mRNA distribution of TRAIL-R3 and TRAIL-R4 was primarily in normal tissues and was absent in the tumor cells tested (14, 15, 21). However, examination of TRAIL receptor mRNA in the human melanoma cell lines found no correlation between the expression of the putative protective TRAIL receptors (TRAIL-R3 and -R4) and resistance or sensitivity to TRAIL. Analysis of a larger panel of human tumors (>60 different lines) has also found no correlation between the expression of the protective TRAIL receptors and resistance to TRAIL (our manuscript in preparation). In contrast, the data presented here clearly demonstrate the importance of intracellular regulators of apoptosis induced by TRAIL. Levels of the apoptosis inhibitor FLIP were highest in the TRAIL-resistant lines and were low or absent in the sensitive lines. Additionally, experiments examining the effect of actinomycin D on FLIP in the TRAIL-resistant melanoma WM 3211 revealed a clear correlation between the increase in sensitivity to TRAIL with decreased FLIP levels. These observations indicate that the expression of the decoy TRAIL receptors does not confer resistance in unmanipulated tumor cells, but, rather, that there are multiple factors that may function to provide protection against the cytotoxic effects of TRAIL.

To date, there has been limited study of the signaling events associated with TRAIL receptor ligation. At the time that TRAIL-R1 and TRAIL-R2 were first described it appeared that the TRAIL receptor system used novel adapter proteins, since neither FADD nor TRADD bound to TRAIL-R1 or TRAIL-R2 (13, 14).

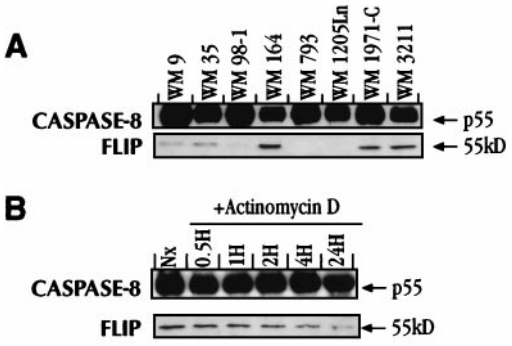

FIGURE 7. Susceptibility to TRAIL-induced apoptosis in melanoma cells is related to intracellular levels of FLIP. *A*, Western blot analysis of caspase-8 and FLIP levels in human melanoma cell lines. Cellular lysates were separated by SDS-PAGE and transferred to nitrocellulose, and caspase-8 and FLIP levels were determined by Western blot analysis. FLIP antiserum was generated as described in *Materials and Methods* and detects the 55-kDa form of human FLIP. *B*, FLIP levels decrease over time in the TRAIL-resistant melanoma WM 3211 compared with those of caspase-8. Flasks containing 5×10^6 cells were incubated with actinomycin D (10 ng/ml) for the indicated times. Cellular lysates were then separated by SDS-PAGE and transferred to nitrocellulose, and caspase-8 and FLIP levels were determined by Western blot analysis.

However, subsequent studies reported contradictory findings by showing direct binding of FADD and TRADD to these two TRAIL receptors and inhibition of TRAIL-induced death with dominant negative forms of FADD and TRADD (16, 28, 29, 40). The discrepancies between these studies may be explained by differences in the relative levels of expression of these molecules in the transfectants used in the experiments. When coupled with data showing that caspase inhibitory peptides block TRAIL-induced apoptosis, it seems evident that many of the death proteases involved in TNF- and FasL-induced apoptosis are also activated by TRAIL receptor ligation. To our knowledge, the data presented here are the first report on the involvement of these molecules in TRAIL-mediated apoptosis in melanomas.

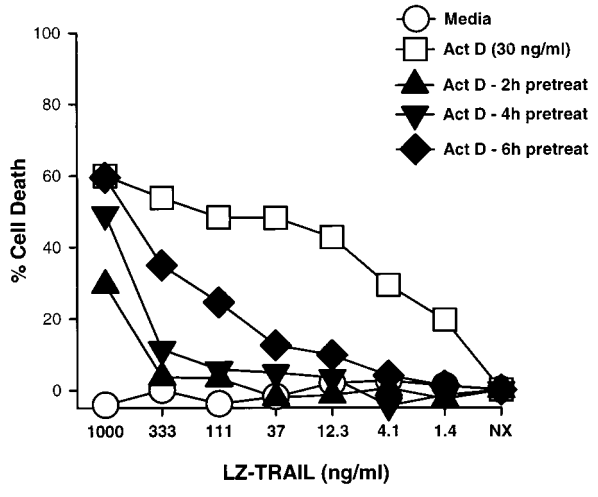

FIGURE 8. TRAIL-induced death following actinomycin D pretreatment correlates with decreasing FLIP levels in WM 3211. Ninety-six-well microtiter plates were seeded with 5×10^4 cells/well and allowed to adhere for at least 6 h. Actinomycin D (30 ng/ml) was added to the cells for 2, 4, or 6 h, and then washed away. LZ-TRAIL was then added, and cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph, but were $<5\%$ for all data points. Experiments were performed at least three separate times with each cell line.

The use of the caspase inhibitors and Abs in this study permitted a finer analysis of the TRAIL receptor signaling cascade. Data presented in Figure 3*A* clearly show that caspase-8 is a proximal component of this pathway, followed by caspase-3. However, it is also likely that other caspases are activated, which could explain why the caspase inhibitor DEVD did not block death as completely as VAD or IETD. A recent study by Schneider et al. (29) showed the potential formation of heterotrimeric TRAIL receptor complexes of TRAIL-R1 and TRAIL-R2, and that ligation of such a mixed receptor may lead to the activation of multiple death cascades within the cell. It is also possible that the caspase-8 homologue caspase-10 (FLICE2/Mch4) (41, 42) may bind to the TRAIL receptor signaling complex, which could then activate other death pathways.

Studies examining human melanomas have defined five major stages in the progression of the disease. These stages are common acquired nevus, dysplastic nevus, radial growth phase (RGP) primary melanoma, vertical growth phase (VGP) primary melanoma, and metastatic melanoma (43). Whereas melanoma cell lines generated from RGP primary melanomas are rarely established, VGP and metastatic melanoma cell lines can be more easily established. The melanoma cell lines used in the experiments presented here were generated from RGP primary melanomas (WM 35 and WM 3211), VGP primary melanomas (WM 98-1 and WM 793), and metastatic melanomas (WM 9, WM 164, and WM 1205Ln) (44). While no correlation could be found between TRAIL sensitivity and the stages from which the cell lines were made (TRAIL-sensitive and TRAIL-resistant cell lines were derived from both primary and metastatic melanomas), our results do suggest that TRAIL could be used to treat human melanoma at each of the various stages and potentially remove any undetected tumors in the body distant from the primary lesion.

Malignant melanoma remains one of the more difficult types of cancer to successfully treat, and with the incidence of melanoma increasing at a rate of approximately 5%/yr over the last 30 yr in Caucasians it continues to be a leading cause of death throughout the world (45). Here we described the mechanism by which TRAIL induces apoptosis in human melanoma cells and a means by which some melanomas remain resistant to TRAIL. Although the results also demonstrate the potential use of TRAIL as a cytotoxic agent against human melanoma, further studies of the mechanisms of TRAIL-mediated cytotoxicity and resistance are required to further assess the potential use of TRAIL as an anticancer therapeutic in vivo.

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