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Functional Analysis of TRAIL Receptors Using Monoclonal Antibodies

Thomas S. Griffith,^{1*} Charles T. Rauch,[†] Pam J. Smolak,[‡] Jennifer Y. Waugh,[§] Norman Boiani,[¶] David H. Lynch,^{*} Craig A. Smith,[‡] Raymond G. Goodwin,[§] and Marek Z. Kubin^{*}

mAbs were generated against the extracellular domain of the four known TNF-related apoptosis-inducing ligand (TRAIL) receptors and tested on a panel of human melanoma cell lines. The specificity of the mAb permitted a precise evaluation of the TRAIL receptors that induce apoptosis (TRAIL-R1 and -R2) compared with the TRAIL receptors that potentially regulate TRAIL-mediated apoptosis (TRAIL-R3 and -R4). Immobilized anti-TRAIL-R1 or -R2 mAbs were cytotoxic to TRAIL-sensitive tumor cells, whereas tumor cells resistant to recombinant TRAIL were also resistant to these mAbs and only became sensitive when cultured with actinomycin D. The anti-TRAIL-R1 and -R2 mAb-induced death was characterized by the activation of intracellular caspases, which could be blocked by carbobenzyloxy-Val-Ala-Asp (OMe) fluoromethyl ketone (zVAD-fmk) and carbobenzyloxy-Ile-Glu(OMe)-Thr-Asp (OMe) fluoromethyl ketone (zIETD-fmk). When used in solution, one of the anti-TRAIL-R2 mAbs was capable of blocking leucine zipper-human TRAIL binding to TRAIL-R2-expressing cells and prevented TRAIL-induced death of these cells, whereas two of the anti-TRAIL-R1 mAbs could inhibit leucine zipper-human TRAIL binding to TRAIL-R1:Fc. Furthermore, use of the blocking anti-TRAIL-R2 mAb allowed us to demonstrate that the signals transduced through either TRAIL-R1 or TRAIL-R2 were necessary and sufficient to mediate cell death. In contrast, the expression of TRAIL-R3 or TRAIL-R4 did not appear to be a significant factor in determining the resistance or sensitivity of these tumor target cells to the effects of TRAIL. *The Journal of Immunology*, 1999, 162: 2597–2605.

The TNF family of cytokines influences a variety of immunological functions, such as cell activation and death (1, 2). TNF and Fas ligand, which are both capable of inducing apoptosis, have received the most intense study due to their participation in autoimmune disorders, activation-induced cell death, immune privilege, and tumor evasion from the immune system (3–7). TNF-related apoptosis-inducing ligand (TRAIL)² (8) is another family member that is capable of inducing apoptosis; TRAIL interacts with five distinct receptors: DR4 (9), DR5/TRAIL-R2 (10–12), TRID (TRAIL receptor without an intracellular domain)/DcR1/TRAIL-R3 (10, 11, 13), TRAIL-R4/DcR2 (14, 15) (hereafter referred to as TRAIL-R1, -R2, -R3, and -R4, respectively), and osteoprotegerin (OPG) (16). Both TRAIL-R1 and TRAIL-R2 contain cytoplasmic death domains, and their overexpression mediates the apoptosis of sensitive cells (9–12). In contrast, neither TRAIL-R3 (which is glycosylphosphatidylinositol-linked) nor TRAIL-R4 (which is a type I membrane protein) contains a complete cytoplasmic death domain, and neither can mediate apoptosis upon ligation with TRAIL (10, 11, 13–15). OPG is a soluble receptor that is capable of binding to TRAIL in vitro

and blocking TRAIL-induced apoptosis (16). Because they bind to TRAIL without directly signaling for cell death, TRAIL-R3, TRAIL-R4, and OPG may be protective receptors, acting either as membrane-bound or soluble antagonistic receptors (10, 11, 15, 16) or via the transduction of an antiapoptotic signal (14). Therefore, the presence or absence of TRAIL-R3, TRAIL-R4, and/or OPG may determine whether a cell is resistant or sensitive, respectively, to TRAIL-induced apoptosis (10, 11, 15, 16).

To further study the function of the death-inducing TRAIL receptors (TRAIL-R1 and -R2) alone or in combination with the receptors that do not induce death (TRAIL-R3 and -R4), mAbs were generated against each of the human TRAIL receptors and used to study a panel of TRAIL-sensitive and -resistant human melanoma cell lines characterized previously for mRNA expression of the four known TRAIL receptors (17). These mAbs were evaluated in terms of their ability to induce apoptosis in the melanoma cell lines (when added in solution or when immobilized on culture plates), to block the binding of TRAIL to melanoma cells expressing TRAIL-R1 and/or -R2, and to inhibit the death of TRAIL-sensitive target cells upon exposure to TRAIL. These mAbs also allowed us to test whether resistance to the cytotoxic effects of TRAIL is influenced by the expression of the putative “decoy receptors”, TRAIL-R3 and/or TRAIL-R4.

Materials and Methods

Cell lines

The human melanoma cell lines (WM 9, 35, 98-1, 164, 793, 1341-D, and 3211) were provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) and cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, and glutamine.

Generation of anti-TRAIL receptor mAb

BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized with a purified fusion protein consisting of the extracellular domain of

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² Abbreviations used in this paper: TRAIL, TNF-related apoptosis-inducing ligand; OPG, osteoprotegerin; PBSA, 3% BSA in PBS; PARP, poly(ADP-ribose) polymerase; HRP, horseradish peroxidase; SA, streptavidin; NFD, nonfat dry milk; Act D, actinomycin D; LZ-huTRAIL, leucine zipper-human TRAIL.

human TRAIL-R1, -R2, -R3, or -R4 coupled to the constant region of human IgG1 (huTRAIL-R:Fc) in Titermax (Cytrx Corporation, Norcross, GA). Mice were boosted three times, and spleen cells were fused with the murine myeloma NS1 in the presence of 50% polyethylene glycol in PBS followed by culture in DMEM/HAT and DMEM/HT selective media. Supernatants from positive wells were tested for the ability to bind the appropriate TRAIL receptor in an ELISA (cell-based ELISA using CV1 cells transfected with TRAIL receptor cDNA) and for reactivity to huTRAIL-R:Fc in Western blots. Hybridomas that produced Abs that bound to huTRAIL-R:Fc but not human IgG1 were cloned by three rounds of limiting dilution. mAb isotypes were determined to be IgG1 (M270, M272, M412, M413, M431, and M445), IgG2a (M271, M273), and IgG2b (M411); all mAbs were purified by protein A affinity chromatography.

Leucine zipper-human TRAIL (LZ-huTRAIL)

The LZ-huTRAIL expression plasmid (12) and the production and purification of LZ-huTRAIL have been described elsewhere (28).

Analysis of LZ-huTRAIL binding to melanoma cells

The surface expression of TRAIL receptor(s) was determined by flow cytometric analysis by measuring the binding of LZ-huTRAIL (17). Briefly, cells were incubated with 10 $\mu\text{g/ml}$ LZ-huTRAIL in 3% BSA in PBS (PBBSA) for 30 min on ice. The cells were washed in PBS, followed by the addition of a biotinylated anti-leucine zipper mAb (M15; 10 $\mu\text{g/ml}$ in 3% PBBSA) for 30 min on ice. After incubation, cells were washed in PBS and then incubated for 30 min on ice with phycoerythrin-conjugated streptavidin (SA) (diluted 1/200 in 3% PBBSA; Sigma, St. Louis, MO). In some cases, cells were preincubated with the TRAIL receptor-specific mAb (10 $\mu\text{g/ml}$ for 30 min on ice) to determine which mAb could inhibit LZ-huTRAIL binding. Cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA).

Inhibition of LZ-huTRAIL binding to TRAIL-R1:Fc

To determine whether any of the anti-TRAIL-R1 mAb could block the binding of LZ-huTRAIL to TRAIL-R1, a modified ELISA was used. We coated 96-well ELISA plates (Corning, Corning, NY) with 1 $\mu\text{g/ml}$ goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) for 4 h at 4°C (all subsequent steps were also performed at 4°C), followed by the addition of TRAIL-R1:Fc (1 $\mu\text{g/ml}$ in 5% nonfat dry milk (NFDM) in PBS-Tween 20 (0.05% v/v)) overnight. Wells were washed with PBS-Tween and subsequently blocked with 5% NFDM in PBS-Tween. After 1 h, the milk was removed and the anti-TRAIL-R1 mAbs were added (10 $\mu\text{g/ml}$ starting concentration diluted in 5% NFDM in PBS-Tween) for 1 h. Wells were washed, followed by the addition of LZ-huTRAIL (1 $\mu\text{g/ml}$ diluted in 5% NFDM in PBS-Tween) for 1 h. After washing, biotinylated anti-leucine zipper mAb (M15; 1 $\mu\text{g/ml}$ diluted in 5% NFDM in PBS-Tween) was added for 1 h. Following the washing of the wells, horseradish peroxidase (HRP)-SA (diluted 1/500 in 5% NFDM in PBS-Tween; Zymed, San Francisco, CA) was added for 1 h. Wells were washed, and colorimetric substrate (TMP microwell peroxidase substrate system; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. Upon color development, H_2SO_4 was added to stop the reaction, and the plate was analyzed on an ELISA plate reader at OD_{450} . The percent inhibition of LZ-huTRAIL binding was determined using the following equation: $100 \times (1 - [\text{experimental group OD} - \text{background OD}] / [\text{untreated group O.D.} - \text{background OD}])$.

Western blotting

TRAIL receptor/OPG blot. Melanoma cells were lysed in PBS containing 1% Nonidet P-40, 0.35 mg/ml PMSF, 9.5 $\mu\text{g/ml}$ leupeptin, and 13.7 $\mu\text{g/ml}$ pepstatin A. The lysates were centrifuged at $14,000 \times g$ to remove cellular debris, and the protein concentrations of the extracts were determined by colorimetric bicinchoninic acid analysis (Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Novex, San Diego, CA), and blocked with 5% NFDM in PBS-Tween 20 overnight at 4°C. The membrane was incubated with TRAIL receptor mAb (1 $\mu\text{g/ml}$ in 5% NFDM in PBS-Tween) or OPG polyclonal Ab (diluted 1/1000 in 5% NFDM in PBS-Tween; provided by Dr. E. Clark, University of Washington, Seattle, WA) for 1 h. After washing, the membrane was incubated for 1 h with an anti-mouse HRP Ab (Amersham, Arlington Heights, IL). Following several washes, the blots were developed by chemiluminescence according to the manufacturer's protocol (Renaissance chemiluminescence reagent, DuPont-New England Nuclear, Boston, MA).

Poly(ADP-ribose) polymerase (PARP) blot. Melanoma cells were incubated with 10 $\mu\text{g/ml}$ TRAIL-R1 and/or -R2 mAb for 30 min, followed by

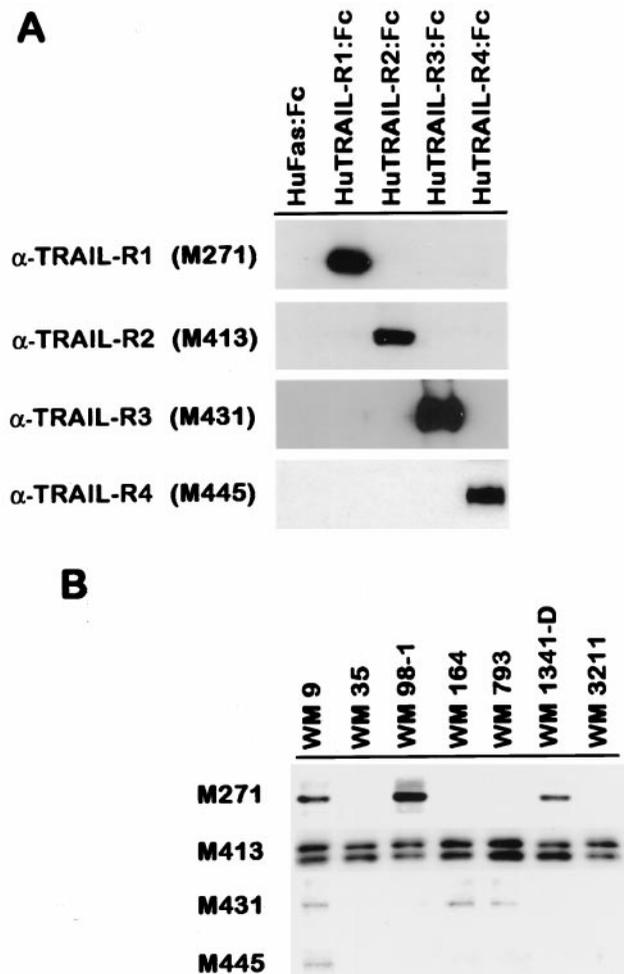


FIGURE 1. Specificity of TRAIL receptor mAb and Western blot analysis of TRAIL receptor expression in human melanoma cell lines. *A*, TRAIL receptor mAb specificity was confirmed using the four different huTRAIL-R:Fc chimeric molecules by Western blot analysis. HuFas:Fc was used as a negative control. *B*, TRAIL receptor expression in human melanoma cells was determined by Western blotting as described in *Materials and Methods*.

the addition of 100 ng/ml LZ-huTRAIL. Cells were lysed after 4 h, and protein concentrations were determined, electrophoresed, and transferred to a nitrocellulose membrane as outlined above. The membrane was incubated with a rabbit anti-PARP Ab (Research Diagnostics, Flanders, NJ) for 1 h. After washing, the membrane was incubated for 1 h with an anti-rabbit HRP Ab (Amersham). Following several washes, the blots were developed by chemiluminescence as described above.

Cytotoxicity assays

Tumor cell sensitivity to TRAIL or TRAIL-R1/-R2 mAb was assayed by incubating the cells in 96-well plates with the indicated amount of purified LZ-huTRAIL, TRAIL receptor-specific mAb, or both in solution or immobilized to the culture plate. In some experiments, mAb to TRAIL-R1 and/or -R2, carbobenzyloxy-Val-Ala-Asp (OMe) fluoromethyl ketone (zVAD-fmk), carbobenzyloxy-Ile-Glu(OMe)-Thr-Asp (OMe) fluoromethyl ketone (zIETD-fmk) (20 μM ; Enzyme Systems Products, Livermore, CA), or actinomycin D (Act D) (30 ng/ml) was added to the culture medium before incubation with LZ-huTRAIL or mAb. Cell death was determined by chromium release (5) or crystal violet staining (18) after 8 or 24 h, respectively, as described previously. Results are presented as the percentage of cell death using the following equations: $100 \times ([\text{experimental group cpm} - \text{background cpm}] / [\text{maximum cpm} - \text{background cpm}])$ for the chromium release experiments or $100 \times (1 - [\text{experimental group OD}] / [\text{untreated group OD}])$ for the crystal violet experiments.

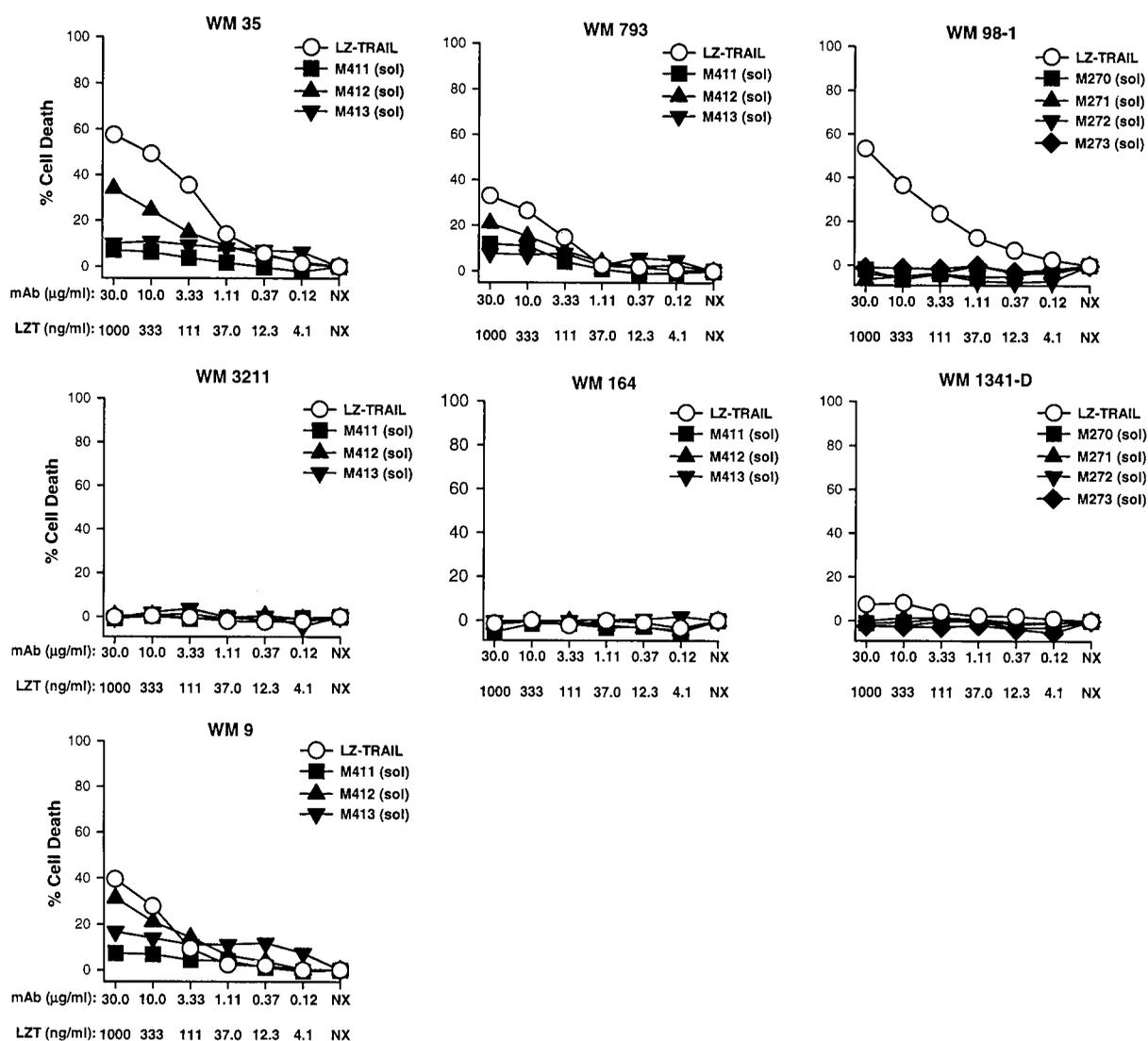


FIGURE 2. Cytotoxic effects of soluble anti-TRAIL-R1 and -R2 mAb on human melanoma cells. A total of $10,000$ ^{51}Cr -labeled melanoma cells were added to each well of 96-well microtiter plates containing soluble anti-TRAIL-R1 mAb (M270, 271, 272, or 273), TRAIL-R2 mAb (M411, 412, or 413), or LZ-huTRAIL. Each value represents the mean of three wells. For clarity, SE bars were omitted from the graph but were $<10\%$ for all data points. Experiments were performed at least three times with each cell line.

Results

Production of TRAIL mAb

Experiments analyzing the TRAIL receptor family have been limited by the lack of receptor-specific Abs. Thus, mAbs were generated that could serve as tools to measure protein expression and dissect individual receptor function. Soluble fusion proteins consisting of the extracellular portion of each human TRAIL receptor coupled to the Fc domain of human IgG1 (huTRAIL-R:Fc) were used to immunize mice and generate mAbs that were reactive with the specific huTRAIL-R:Fc but not human IgG1 by ELISA (data not shown) or Western blotting (Fig. 1A). In addition, the anti-TRAIL receptor mAb only reacted with the appropriate CV1/EBV-encoded nuclear Ag cells transfected with cDNAs for each of the four TRAIL receptors in a slide binding assay (data not shown), further demonstrating receptor specificity and no cross-reactivity with the other three TRAIL receptors.

These mAbs were then used to screen a panel of human melanoma cell lines by Western blot analysis (Fig. 1B); this panel had

been characterized previously for its expression of TRAIL receptor mRNA (17). The results of this screening identified several pairs of TRAIL-sensitive and -resistant cell lines as having the same TRAIL receptor expression pattern. For example, WM 35 (sensitive) and WM 3211 (resistant) only expressed TRAIL-R2. TRAIL-R2 and -R3 were expressed by WM 793 (sensitive) and WM 164 (resistant), whereas WM 98-1 (sensitive) and WM 1341-D (resistant) expressed both TRAIL-R1 and TRAIL-R2. Lastly, the TRAIL-sensitive line WM 9 was found to express all four TRAIL receptors. The seven melanoma cell lines were also analyzed for OPG expression, and none were found to express this protein (data not shown). The doublet seen in the blot with the anti-TRAIL-R2 mAb (M413) is consistent with previous observations predicting two variants of TRAIL-R2 resulting from an 11-aa deletion at the amino terminus (12). These results on TRAIL receptor protein expression are concordant with the RT-PCR results examining TRAIL receptor mRNA expression (17) and extend the earlier observed lack of correlation between decoy TRAIL receptor (TRAIL-R3 and -R4) mRNA expression and TRAIL resistance.

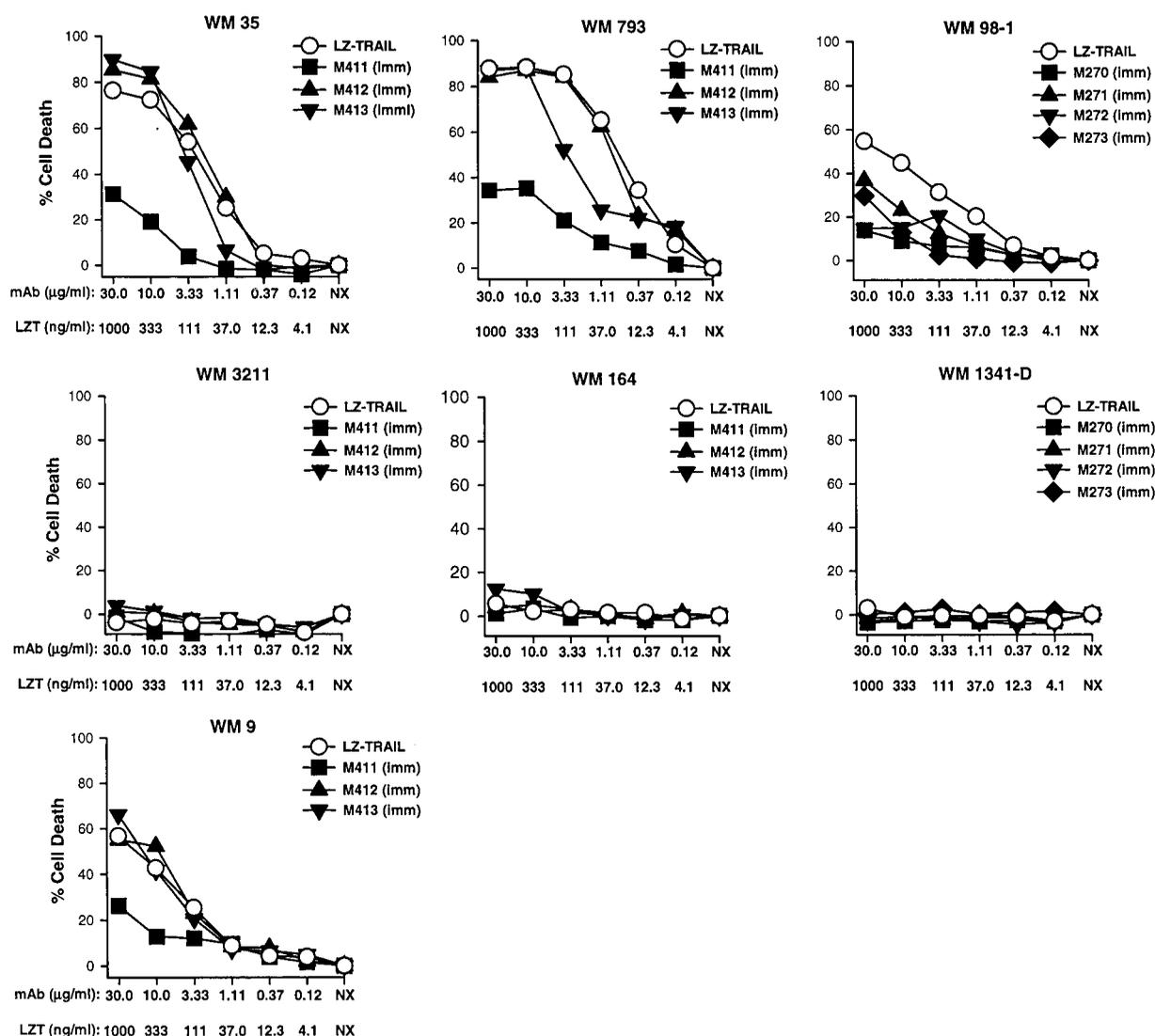


FIGURE 3. Cytotoxic effects of immobilized anti-TRAIL-R1 and -R2 mAb on human melanoma cells. For immobilization, 96-well microtiter plates were incubated overnight at 4°C with the different mAbs diluted in 0.1 M sodium bicarbonate buffer. After washing wells with PBS, 10^4 ^{51}Cr -labeled melanoma cells were added to each well of microtiter plates containing immobilized anti-TRAIL-R1 mAb (M270, 271, 272, or 273), TRAIL-R2 mAb (M411, 412, or 413), or LZ-huTRAIL. Each value represents the mean of three wells. For clarity, SE bars were omitted from the graph but were <10% for all data points. Experiments were performed at least three times with each cell line.

Anti-TRAIL-R1 and -R2 mAb-induced apoptosis

The results of previous studies using Fas-specific mAbs have demonstrated that although they may act as antagonists when added in soluble form, these mAbs can act as potent agonists when appropriately cross-linked (19). Thus, it was of interest to determine whether the anti-TRAIL-R1 and -R2 mAbs acted in a similar fashion. None of the anti-TRAIL-R1 and only one of the anti-TRAIL-R2 mAbs (M412) tested were able to induce lysis of TRAIL-sensitive melanoma cells when added to cultures in solution in an 8-h ^{51}Cr release assay, and the lysis induced by M412 was weak (Fig. 2). Moreover, the TRAIL-resistant melanoma lines (WM 164, 1341-D, and 3211) were also resistant to soluble anti-TRAIL-R1 and -R2 mAb-induced lysis. Interestingly, all of the anti-TRAIL-R2 and two of the TRAIL-R1 mAbs that failed to or minimally induced lysis of TRAIL-sensitive melanoma cells when added in solution displayed increased lytic ability when immobilized to the culture plate (Fig. 3). Again, this lysis was only seen with the TRAIL-sensitive melanoma lines and not with the

TRAIL-resistant lines. Thus, these results demonstrate that melanoma cell sensitivity to anti-TRAIL receptor mAb is comparable with that of TRAIL, and further suggest that the nonsignaling TRAIL-R3 and -R4 do not play a major role in resistance to TRAIL-induced apoptosis.

Inhibition of TRAIL binding and TRAIL-induced apoptosis by anti-TRAIL-R1 and -R2 mAb

Three methods were employed to determine whether the anti-TRAIL-R1 and -R2 mAbs were able to block LZ-huTRAIL binding to the appropriate receptors and prevent TRAIL-induced apoptosis. Using the TRAIL-sensitive melanoma cell lines shown to express only TRAIL-R2 (WM 35), TRAIL-R1 and -R2 (WM 98-1), or TRAIL-R2 and -R3 (WM 793), the anti-TRAIL-R2 mAbs were first assessed for their ability to block the binding of LZ-huTRAIL as measured by flow cytometry. Cells were incubated with the anti-TRAIL-R2 mAb, control mAb, or no mAb for 20

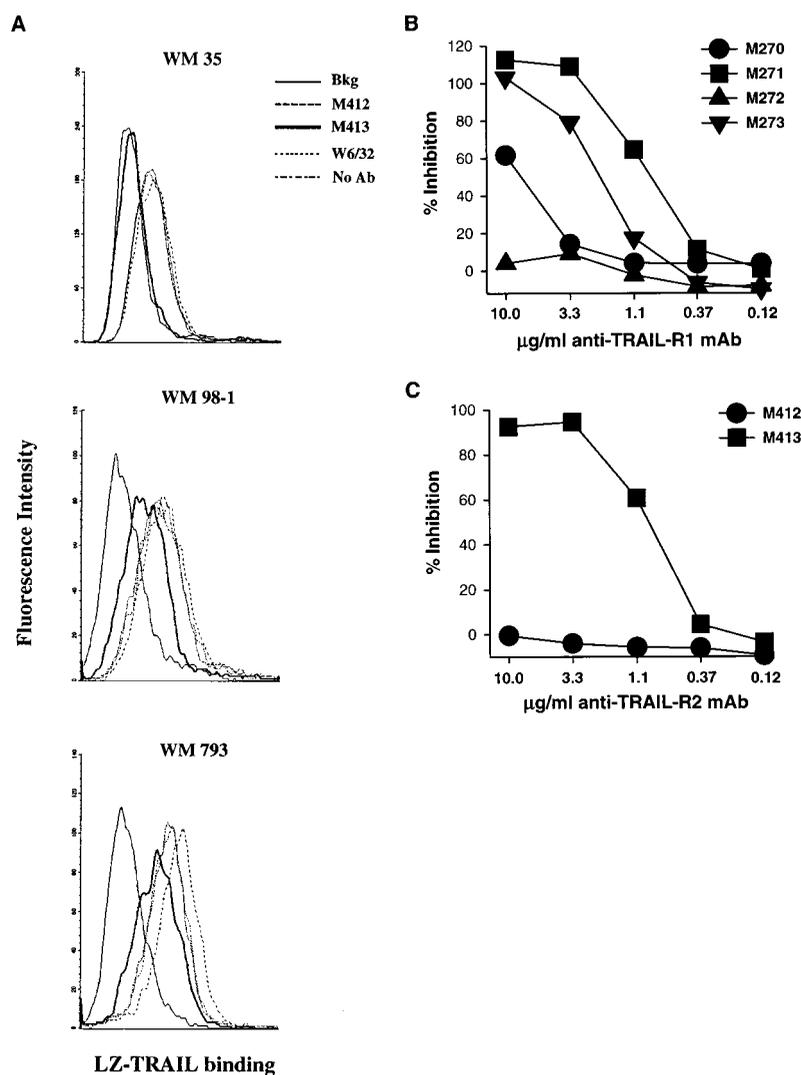


FIGURE 4. Inhibition of LZ-huTRAIL binding by anti-TRAIL-R1 and -R2 mAb. *A*, WM 35 (TRAIL-R2⁺), WM 98-1 (TRAIL-R1⁺, -R2⁺), and WM 793 (TRAIL-R2⁺, -R3⁺) cells were incubated for 20 min with TRAIL-R2 mAb M412, M413, control mAb W6/32, or no Ab. Cells were washed and subsequently incubated with LZ-huTRAIL, biotinylated mouse anti-leucine zipper Ab M15, and phycoerythrin-conjugated SA as described in *Materials and Methods*. Cells were analyzed by flow cytometry to measure LZ-huTRAIL binding. *B* and *C*, Inhibition of LZ-huTRAIL binding to TRAIL-R1:Fc and TRAIL-R2:Fc, respectively, by the receptor-specific mAb as described in *Materials and Methods*.

min, followed by incubation with LZ-huTRAIL. Secondary reagents were then added to measure the level of LZ-huTRAIL binding to the cells. M413 mAb was found to completely inhibit LZ-huTRAIL binding to WM 35 (Fig. 4A). However, as expected M413 could not fully inhibit LZ-huTRAIL binding to either WM 98-1 or WM 793 cells, which express additional TRAIL receptors. The anti-TRAIL-R2 mAb, M412, was unable to inhibit LZ-huTRAIL binding as seen with M413.

To determine whether any of the anti-TRAIL-R1 mAb also displayed a similar ability to block LZ-huTRAIL binding to TRAIL-R1, a modified ELISA was used with the TRAIL-R1:Fc fusion protein. This assay was used because none of the melanoma cell lines available expressed TRAIL-R1 in the absence of TRAIL-R2. Two of the anti-TRAIL-R1 mAbs (M271 and M273) significantly inhibited the binding of LZ-huTRAIL to TRAIL-R1:Fc (Fig. 4B). In addition, M270 could also inhibit LZ-huTRAIL binding, but only at the highest Ab concentration. To confirm that this assay accurately measured the inhibition of LZ-huTRAIL binding, a similar experiment was performed using TRAIL-R2:Fc and the anti-TRAIL-R2 mAbs M412 and M413 that are shown in Fig. 4A. Indeed, only M413 inhibited LZ-huTRAIL binding to TRAIL-R2:Fc, whereas M412 did not (Fig. 4C). Collectively, these results demonstrate that two of the anti-TRAIL-R1 mAbs and one of the anti-TRAIL-R2 mAbs are capable of inhibiting the binding of LZ-huTRAIL to their respective receptors.

Next we tested the capability of the M271 and M413 mAbs to act as antagonists by protecting these TRAIL-sensitive melanoma cell lines from TRAIL-induced death. The data demonstrate that the addition of M413 significantly decreased the amount of death seen following LZ-huTRAIL addition to WM 35 and WM 793 target cells, both of which express TRAIL-R2 but not TRAIL-R1 (Fig. 5A). Whereas the preincubation of M412 was unable to prevent the TRAIL-induced death of the melanoma cells, there was an increase in cell death upon incubation with M412 and LZ-huTRAIL. This increase probably resulted from the cytotoxic activity of both M412 and LZ-huTRAIL, because M412 was the only anti-TRAIL-R2 mAb that demonstrated this activity when in solution (Fig. 2). The fact that no such inhibition was observed with WM 98-1 target cells suggests that death in this case was mediated via TRAIL-R1 signaling. Based on the results from Fig. 4B demonstrating that the anti-TRAIL-R1 mAb M271 could inhibit LZ-huTRAIL binding to TRAIL-R1:Fc, we tested whether the coin-cubation of M271 and M413 could inhibit the TRAIL-induced death of WM 98-1. Whereas no inhibition of death was seen with the individual mAb, there was significant inhibition when both mAbs were added before LZ-huTRAIL (Fig. 5B).

Western blot analysis of PARP cleavage in these three lines found that M413 could prevent this apoptotic event in WM 35 and WM 793 cells but not in WM 98-1 cells (Fig. 6). Thus, these results demonstrate that the addition of M413 mAb in solution can

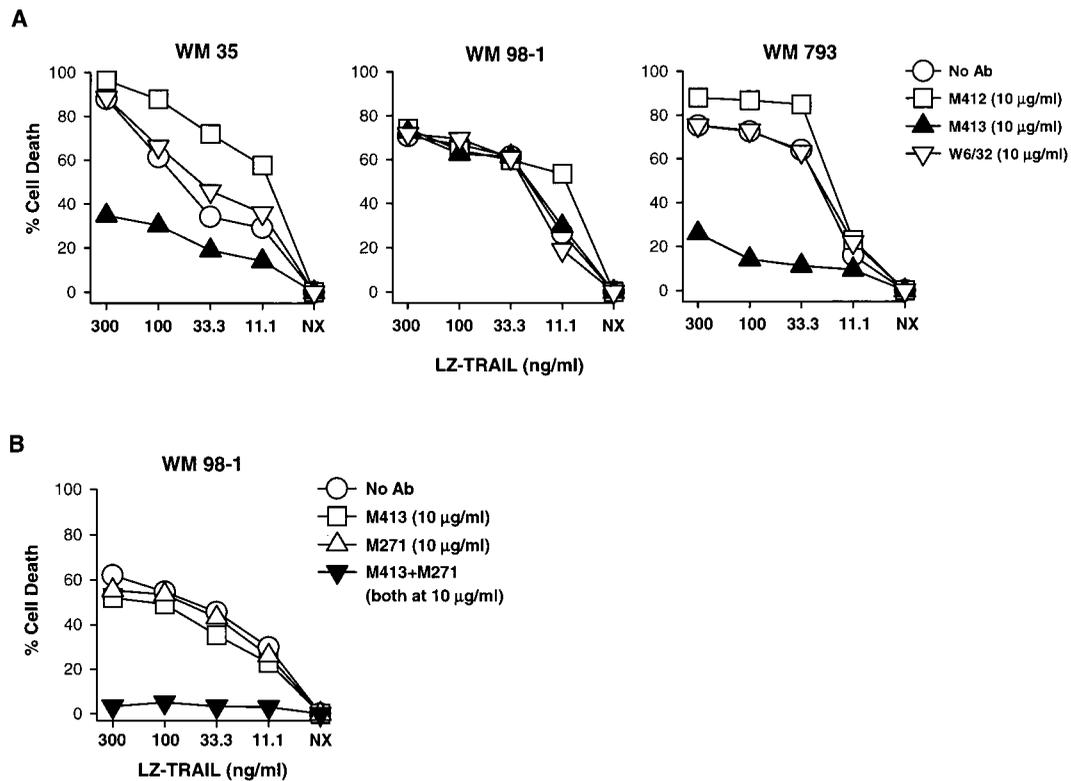


FIGURE 5. Inhibition of LZ-huTRAIL-induced death with soluble M413 and M271. *A*, The 96-well microtiter plates were seeded with 5×10^4 WM 35 (TRAIL-R2⁺), WM 98-1 (TRAIL-R1⁺, -R2⁺), or WM 793 (TRAIL-R2⁺, -R3⁺) cells/well and allowed to adhere for ≥ 6 h. M412 (10 µg/ml), M413 (10 µg/ml), W6/32 (10 µg/ml), or medium was added to each well 30 min before adding LZ-huTRAIL. *B*, LZ-huTRAIL-induced death of WM 98-1 (TRAIL-R1⁺, -R2⁺) following treatment with soluble M413 (10 µg/ml), M271 (10 µg/ml), or M413 and M271 (10 µg/ml each). Protection against LZ-huTRAIL-induced death was only provided by pretreating with both M413 and M271. Each value represents the mean of three wells. For clarity, SE bars were omitted from the graph but were $<10\%$ for all data points. Experiments were performed three times with each cell line.

block LZ-huTRAIL binding to TRAIL-R2 and inhibit TRAIL-induced death in cells expressing TRAIL-R2 in the absence but not the presence of TRAIL-R1. The results also show that LZ-huTRAIL binding to TRAIL-R1 can be blocked by two of the anti-TRAIL-R1 mAbs (M271 and M273), and that the addition of both M271 and M413 can inhibit the TRAIL-induced death of cells expressing both TRAIL-R1 and -R2.

Resistance to anti-TRAIL receptor mAb-induced killing is mediated by an intracellular inhibitor of caspase activation

Previous studies examining TRAIL-induced apoptosis revealed the activation of several caspases and determined that TRAIL-resistant

cells could be converted to TRAIL-sensitive upon the addition of Act D (17). To determine whether these events also occurred during anti-TRAIL-R1 and -R2 mAb-induced death, the sensitive melanoma line WM 98-1 was incubated with immobilized LZ-huTRAIL, M271, or M413 mAb along with the caspase inhibitors zVAD or zIETD (20–23). The death induced by each reagent was inhibited by the addition of zVAD or zIETD (Fig. 7). Next, the TRAIL-resistant lines WM 164 and WM 1341-D were incubated with immobilized LZ-huTRAIL, M413, or M271 in the presence or absence of Act D. Significant cell death was induced only in the presence of Act D, whereas minimal death occurred in its absence (Fig. 8). Similar results were also seen when other anti-TRAIL-R1 and -R2 mAbs were used with Act D (data not shown). These results indicate that mAb cross-linking of the death-inducing TRAIL receptors does not always lead to apoptotic cell death, and that the mAb-induced death results from the activation of the signaling cascade seen during TRAIL-induced apoptosis.

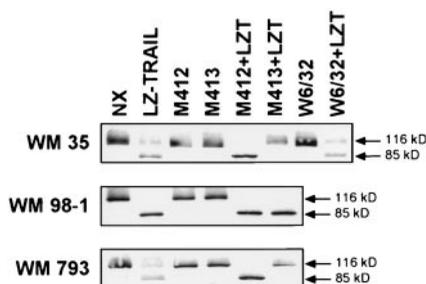


FIGURE 6. Soluble M413 inhibits PARP cleavage in TRAIL-R2- but not TRAIL-R1/-R2-expressing melanoma cells. Flasks containing 5×10^6 WM 35 (TRAIL-R2⁺), WM 98-1 (TRAIL-R1⁺, -R2⁺), or WM 793 (TRAIL-R2⁺, -R3⁺) cells were incubated with W6/32 (10 µg/ml), M412 (10 µg/ml), or M413 (10 µg/ml) for 30 min. LZ-huTRAIL (100 ng/ml) was then added to the cells for 4 h, after which cell lysates were prepared and PARP cleavage was determined by Western blot analysis.

Discussion

The four TRAIL receptors bind TRAIL with comparable affinities (14), and when this fact is combined with the observation that TRAIL-R3 and -R4 are primarily expressed in normal tissues and absent in tumor cells (10, 11, 15), one could conclude that the expression of either or both of these receptors would provide resistance to TRAIL-induced apoptosis. However, it is important to remember that the initial reports examining the distribution of the different TRAIL receptors were at the transcript level via Northern blot analysis (9–15). Although these reports indicated the cells and tissues that could potentially express one or more of the TRAIL

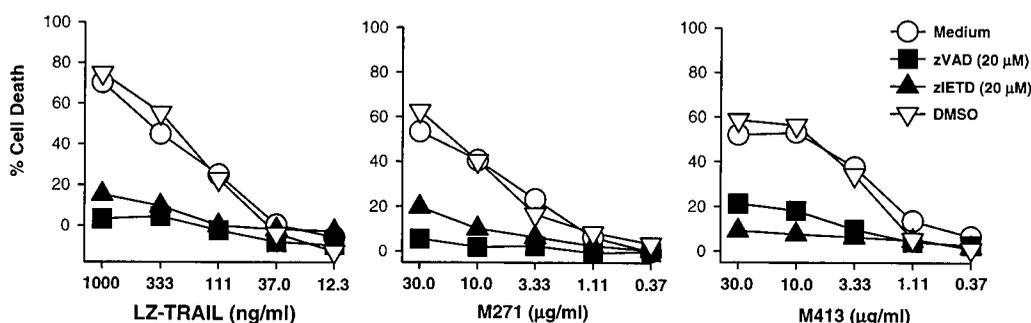


FIGURE 7. Inhibition of immobilized anti-TRAIL-R1 and -R2 mAb-induced apoptosis by caspase-inhibiting peptides. We incubated 96-well microtiter plates overnight at 4°C with M271 and M413 diluted in 0.1 M sodium bicarbonate buffer. After washing wells with PBS, 10⁴ ⁵¹Cr-labeled WM 98-1 melanoma cells were added to each well of microtiter plates containing zVAD (20 μM), zIETD (20 μM), DMSO, or media and immobilized LZ-huTRAIL, M271, or M413. Each value represents the mean of three wells. For clarity, SE bars were omitted from the graph but were <10% for all data points. Experiments were performed at least three times with each cell line.

receptors, it was unknown whether these same cells and tissues actually expressed the predicted repertoire of TRAIL receptor proteins. More importantly, perhaps, it was uncertain whether the natural expression levels of TRAIL-R3 and -R4 were responsible for mediating resistance to TRAIL-induced apoptosis, as originally suggested (10, 11, 13–15).

Using mAbs against the four TRAIL receptors, we were able to survey a panel of human melanoma cell lines for TRAIL receptor protein expression; this panel had been characterized previously for TRAIL receptor mRNA by RT-PCR (17). We found the expression of the TRAIL receptor proteins to be completely concordant with the mRNA expression, such that no correlation between the expression of the presumed decoy TRAIL receptor proteins and resistance or sensitivity to TRAIL was observed. Although a lower amount of TRAIL-R3 and -R4 protein was detected in the cells expressing these receptors as compared with TRAIL-R1 and -R2, it is difficult to determine whether this low protein level prevents these TRAIL receptors from conferring resistance to TRAIL-mediated apoptosis. This uncertainty is largely due to the fact that it is not currently known how the different TRAIL receptors interact with one another on the cell surface, nor is it known how much TRAIL-R3 or -R4 must be present on the surface of normal (i.e., untransfected) cells to disrupt the formation of TRAIL-R1 or

-R2 signaling complexes to provide protection from the cytotoxic effects of TRAIL, as initially predicted in overexpression experiments (10, 11, 13–15). Although TRAIL-R3 or -R4 may act as a decoy when overexpressed subsequent to transfection, the results presented here suggest that they do not appear to be protective under physiological conditions. The mAbs used in this paper will permit further study of these issues.

In addition to analyzing the expression of the different TRAIL receptors, the production of agonistic mAbs that were reactive specifically against the death-inducing receptors TRAIL-R1 and -R2 made it possible to bypass the proposed protective nature of TRAIL-R3 and -R4, thereby examining cell sensitivity solely through TRAIL-R1 or -R2 cross-linking. The decoy receptor hypothesis proposed that the inclusion of these protective receptors into a trimerized receptor complex bound by TRAIL would prevent death-inducing signaling complex formation and thus confer resistance to TRAIL-induced apoptosis (10, 11, 15). The testing of this decoy receptor hypothesis with anti-TRAIL-R1 or -R2 mAb demonstrated that mAb-induced apoptosis only occurred in the melanoma cell lines that were also sensitive to the natural ligand TRAIL, regardless of the presence or absence of TRAIL-R3 and/or -R4. Moreover, the TRAIL-resistant cells only became sensitive to

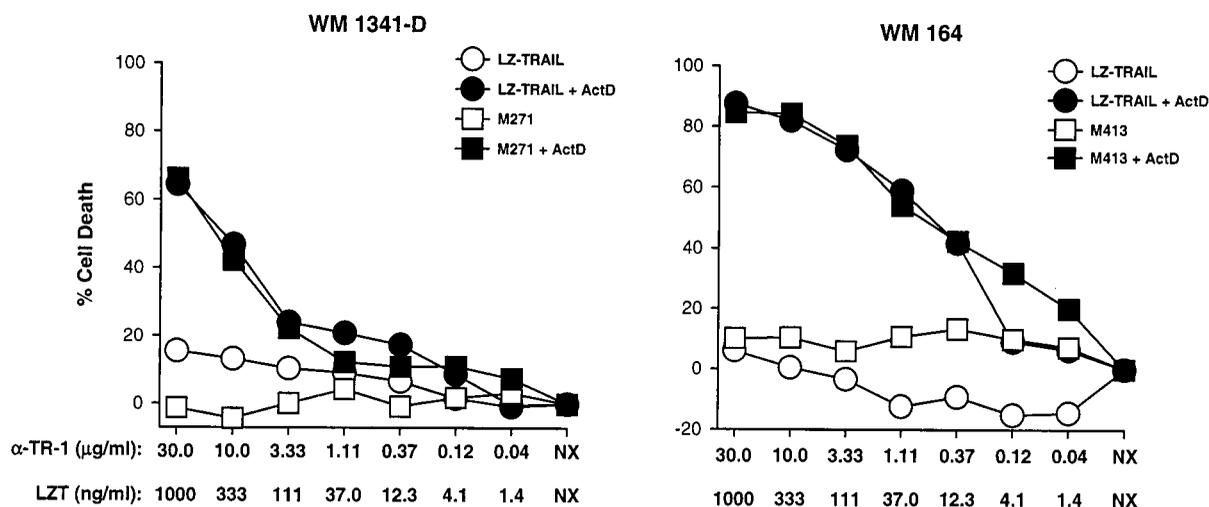


FIGURE 8. Act D makes resistant melanomas sensitive to both recombinant TRAIL and TRAIL receptor mAb. The 96-well microtiter plates were seeded with WM 1341-D or WM 164 (5 × 10⁴ cells/well) and allowed to adhere for ≥6 h. Act D (30 ng/ml) was added to each well immediately before adding LZ-huTRAIL. Each value represents the mean of three wells. For clarity, SE bars were omitted from the graph but were <10% for all data points. Experiments were performed three times with each cell line.

the anti-TRAIL-R1 and -R2 mAbs when cultured with Act D, further supporting the idea that intracellular regulation of TRAIL-induced apoptosis plays a greater protective role in TRAIL sensitivity or resistance. We have recently found a correlation between high levels of the antiapoptotic protein FADD-like IL-1 β -converting enzyme-inhibitory protein (24) in these human melanoma cell lines and resistance to TRAIL-induced apoptosis (17). Although these data suggest a role for FADD-like IL-1 β -converting enzyme-inhibitory protein in the susceptibility of tumor cells to TRAIL, the potential contribution of other intracellular molecules in regulating TRAIL receptor signaling and apoptosis cannot be excluded.

To our knowledge, the mAbs used in this study have also led to the first demonstration that naturally expressed TRAIL-R1 can signal for cell death. Previous studies with TRAIL-R1 relied on overexpression of the receptor on transfected cells to demonstrate the apoptosis-inducing nature of this TRAIL receptor (9). The anti-TRAIL-R1 mAbs are able to directly induce apoptotic cell death in TRAIL-R1-expressing, TRAIL-sensitive melanoma cells. Because none of the cell lines available express TRAIL-R1 alone, we could not determine whether any of the TRAIL-R1 mAbs are capable of blocking LZ-huTRAIL binding to the cells and inhibiting cell death as seen with M413. However, the use of an ELISA-based assay with TRAIL-R1:Fc found that two of the anti-TRAIL-R1 mAbs could significantly inhibit LZ-huTRAIL binding in this setting, suggesting that the mAbs against TRAIL-R1 could inhibit the TRAIL-induced apoptosis of cells that only express TRAIL-R1 or be combined with M413 to prevent the death of cells expressing TRAIL-R1 and -R2. This finding proved to be correct when examining the ability of the anti-TRAIL-R1 mAb M271 or anti-TRAIL-R2 mAb M413 to inhibit the TRAIL-induced death of WM 98-1 (TRAIL-R1⁺ and -R2⁺) melanoma cells when added individually or together. Protection was only seen when both blocking mAbs were added before adding LZ-huTRAIL. This protection was also seen when the other blocking anti-TRAIL-R1 mAbs were combined with M413 (data not shown).

Recent findings have demonstrated that another receptor, OPG, is capable of binding TRAIL (16). OPG is a secreted TNF receptor-related protein that can block osteoclastogenesis in vitro and increase bone density and act as a protective agent against ovariectomy-associated bone loss in vivo when overexpressed (25). Thus, soluble OPG may function as a competitive inhibitor of its cognate TNF family ligand, preventing the ligand from binding to its cellular receptor. In the search for a ligand for OPG, it was determined that OPG binds TRAIL and can inhibit the TRAIL-induced apoptosis of Jurkat cells, even though the binding affinity of OPG is slightly weaker than the affinities of the TRAIL receptors for TRAIL (16). Further in vitro studies with OPG have found that TRAIL can inhibit the osteoclastogenic ability of OPG (16). These two observations suggest that OPG and TRAIL may function to inhibit one another. The release of OPG from a cell may also serve as a natural inhibitor of TRAIL-induced death, similar to the protection conferred by soluble Fas (26, 27). It is unlikely that OPG is involved in determining TRAIL resistance in human melanoma cells, because none of the seven human melanoma cell lines used in this study were found to express OPG protein as determined by Western blot analysis (data not shown). However, OPG may play a role in preventing TRAIL-induced apoptosis in normal tissues or other tumor cell types. Additional studies will need to be performed to address these possibilities.

The existence of four TRAIL receptors, two with the ability to signal for apoptosis (TRAIL-R1 and -R2) and two without (TRAIL-R3 and -R4), as well as the soluble TRAIL-binding protein OPG demonstrates the biological complexity of this receptor-ligand system. The results presented here show that TRAIL-R1 or

-R2 ligation does not always lead to death and suggest that TRAIL-induced apoptosis is not regulated simply through either the competitive binding of TRAIL by TRAIL-R3 or -R4 or by the transduction of protective signals mediated by either of these receptors. It seems likely that multiple factors function together to provide resistance against the cytotoxic effects of TRAIL, the most important of which appears to be intracellular regulation of caspase activation (17). Although many questions still remain unanswered regarding the biological function of each of these TRAIL receptors and the signals generated upon their ligation, the TRAIL receptor-specific mAbs described here will serve as valuable tools in the future study of these receptors.

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