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# Relation of TNF-related Apoptosis-inducing Ligand (TRAIL) Receptor and FLICE-inhibitory Protein Expression to TRAIL-induced Apoptosis of Melanoma[1]

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## ABSTRACT

**Past studies have shown that apoptosis mediated by TNF-related apoptosis-inducing ligand (TRAIL) is regulated by the expression of two death receptors [TRAIL receptor 1 (TRAIL-R1) and TRAIL-R2] and two decoy receptors (TRAIL-R3 and TRAIL-R4) that inhibit apoptosis. In previous studies, we have shown that TRAIL but not other members of the tumor necrosis factor family induce apoptosis in approximately two-thirds of melanoma cell lines. Here, we examined whether the expression of TRAIL-R at the mRNA and protein level in a panel of 28 melanoma cell lines and melanocytes correlated with their sensitivity to TRAIL-induced apoptosis. We report that at least three factors appear to underlie the variability in TRAIL-induced apoptosis. (*a*) Four of nine cell lines that were insensitive to TRAIL-induced apoptosis failed to express death receptors, and in two instances, lines were devoid of all TRAIL-Rs. Southern analysis suggested this was due to loss of the genes for the death receptors. (*b*) Despite the presence of mRNA for the TRAIL-R, some of the lines failed to express TRAIL-R protein on their surface. This was evident for TRAIL-R1 and more so for the TRAIL decoy receptors TRAIL-R3 and -R4. Studies on permeabilized cells revealed that the receptors were located within the cytoplasm and redistribution from the cytoplasm may represent a posttranslational control mechanism. (*c*) Surface expression of TRAIL-R1 and -R2 (but not TRAIL-R3 and -R4) showed an overall correlation with TRAIL-induced apoptosis. However, certain melanoma cell lines and clones were relatively resistant to TRAIL-induced apoptosis despite the absence of decoy receptors and moderate levels of TRAIL-R1 and -R2 expression. This may indicate the presence of inhibitors within the cells, but resistance to apoptosis could not be correlated with expression of the caspase inhibitor FLICE-inhibitory protein. mRNA for another TRAIL receptor, osteoprotegerin, was expressed in 22 of the melanoma lines but not on melanocytes. Its role in induction of apoptosis remains to be studied. These results appear to have important implications for future clinical studies on TRAIL.**

## INTRODUCTION

Several members of the TNF[4] family have been shown to induce apoptosis in susceptible cells by activation of the caspase pathway (1–3). Induction of apoptosis appears to be restricted to receptors that contain "death domains," such as those for FasL, TNF-$\alpha$, TRAIL/apo-2 (4, 5), and apo-3 ligand (6). Receptors for the latter, also known as TNF receptor-related apoptosis-mediated protein, DR3, and LARD (7, 8), appear to be mainly related to induction of apoptosis in lymphocytes. In contrast, TRAIL appears to be able to induce apoptosis in a wide range of transformed cell lines but not normal cells (4, 5). TRAIL can induce apoptosis by interaction with two receptors, referred to as DR4 (TRAIL-R1; Refs. 9 and 10) and DR5/TRAIL-R2/TRICK 2 (11–13). These receptors were found to be widely expressed on normal tissues, which are believed to be protected from apoptosis by two additional receptors, TRAIL-R3/TRID/DcR1/LIT (14–17) and TRAIL-R4/DcR2/TRUNDD (18, 19). TRAIL-R3 and -R4 inhibit apoptosis either by acting as decoy receptors or by providing inhibitory signals, perhaps via activation of NF-$\kappa$B (3, 20). A fifth receptor, OPG, exists in a secreted form and appears to inhibit TRAIL-induced apoptosis by competitive inhibition of TRAIL binding to the death receptors TRAIL-R1 and -R2 (21).

In addition to the control of TRAIL-induced apoptosis by expression of "death-inducing" and "decoy" receptors on cells, apoptosis induced by TRAIL and other TNF members may be regulated by inhibitory proteins that bind to Fas-associated death domain or other proteins in the caspase pathway. Cellular FLIP appeared to be more active against TRAIL than against Fas-induced apoptosis of Jurkat cells and was detected in both melanoma cell lines and cutaneous melanoma metastases (22). In addition to FLIP, two other IAPs (IAP1 and IAP2) that are induced by activation of NF-$\kappa$B may have similar roles in blocking apoptosis by inhibition of caspase 8 (23).

We have shown previously that melanoma cells express receptors for CD40 ligand (24), TNF-$\alpha$, and FasL (25), but ligands for these receptors were not able to induce apoptosis in the melanoma cells. In contrast, TRAIL was able to induce varying degrees of apoptosis in approximately two-thirds of the melanoma lines tested (26). Here, we have examined a large panel of melanoma cells for expression of TRAIL-R at the mRNA and protein level to determine whether the variable induction of apoptosis in melanoma by TRAIL can be related to the expression of the different types of TRAIL-R. We have also examined whether the expression of FLIP protein may be an important determinant of sensitivity to TRAIL-induced apoptosis. The results confirm the importance of TRAIL-R1 or -R2 expression for induction of apoptosis by TRAIL, but the expression of the decoy receptors or FLIP protein did not appear to be related to TRAIL-induced apoptosis of melanoma cells.

## MATERIALS AND METHODS

**Cell Lines.** Melanoma cell lines with the prefix Mel were isolated from fresh surgical biopsies from patients attending the Sydney and Newcastle Melanoma Units and established in the laboratory. JS, FH, WB, CV, JC, MS, LT, KN, JB, AT, GL, KM3, and RMu were from lymph nodes. MC, JF, and MM were from skin. RM, JG, MI, and SD were from bowel. The cell lines had been in culture for 2–6 months at the time of these studies. A2058 was from brain. MM200, Me1007, Me10538, Me4405, and IgR3 were from primary melanoma. The derivation of MM200, Me1007, Me10538, and Me4405 are described elsewhere (24). A2058 was established from a brain metastasis and was provided by Dr. T. Todaro (Bristol-Myers Squibb; Seattle, WA). Me3.1 was a kind gift from Dr. T. Boon (Ludwig Institute, Brussels, Belgium). All melanoma cell lines were positive for Tyrosinase and MART-1 mRNA by RT-PCR tests described elsewhere (27), except for Mel SP, which was positive for Tyrosinase but not MART-1. All cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia).

**MAbs and Recombinant Proteins.** Recombinant human TRAIL (lot no. 6321-19), prepared as described elsewhere (4), was supplied by Immunex (Seattle, WA). The preparation was supplied as a leucine zipper fusion protein, which required no further cross-linking for maximal activity. The MAbs against TRAIL-R1 (IgG2a hu TR1-M271; lot no. 7136-07), TRAIL-R2 (IgG1

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[4] The abbreviations used are: TNF, tumor necrosis factor; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; NF-$\kappa$B, nuclear factor $\kappa$B; OPG, osteoprotegerin; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; MAb, monoclonal antibody; Ab, antibody; RT-PCR, reverse transcription-PCR.

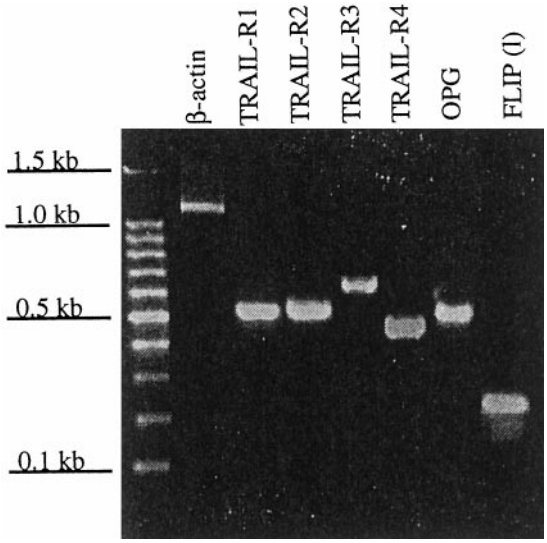


Fig. 1. PCR products of the TRAIL receptors and FLIP. The product sizes (in bp) were as follows: β-actin, 1126; TRAIL R1–R4 and OPG, 506, 502, 612, 453, and 486, respectively; $FLIP_L$ and $FLIP_S$, 226 and 228, respectively.

hu TRAIL-R2-M413; lot no. 5274-96), TRAIL-R3 (IgG1 hu TR3-M430; lot no. 7313-17), and TRAIL-R4 (IgG1 hu TR4-M444; lot no. 7136-15) were also supplied by Immunex. Their specificities and functions are described elsewhere (28). The rabbit Ab against FLIP, AL129 (lot no. P3), was prepared as described elsewhere (22) and was kindly supplied by Dr. J. Tschopp (Ludwig Institute, Lausanne, Switzerland). Isotype control MAbs used were the ID4.5 (IgG2a) MAb against *Salmonella typhi*, supplied by Dr. L. Ashman (IMVS, Adelaide, South Australia, Australia), and the 107.3 IgG1 MAb (PharMingen, San Diego, CA). The isotype control used for studies on FLIP was purified rabbit IgG, supplied by Sigma Chemical Co. (St. Louis, MO).

**RT-PCR Detection of TRAIL, TRAIL-R, OPG, and FLIP.** Total RNA was isolated from $1 \times 10^6$ melanoma cells using RNAzol B RNA extraction (Biotecx, Friendswood, TX). The first-strand cDNA was synthesized with a first strand cDNA synthesis kit using Moloney murine leukemia virus reverse transcriptase with oligo(dT) primers (Novagen, Milwaukee, WI). Of the resultant cDNA, 1 μl was used in the 10-μl PCR mix, containing 0.5 μM each relevant primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, 1× reaction buffer IV (Advanced Biotechnologies, Silver Spring, MD), and 0.5 units of thermostable DNA polymerase (Advanced Biotechnologies). The housekeeping gene *β-actin* was used as a control. The primer sequences for TRAIL-R are as described elsewhere (29). The primer sequences for OPG were designed using the Australian National Genomic Information Service (30). Primers for FLIP short and long forms of FLIP were designed using the Primer 3 program database.[5] The sequences for the relevant primers were as follows: *β-actin*, 5′- ATGGATGATGATATCGCCGCG-3′ and 5-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3′; TRAIL-R1, 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′ and 5′-TCCAAGGACACGGCAGAGCCTGTGCCAT-3′; TRAIL-R2, 5′-GCCTCATGGACAATGAGATAAAGGTGGCT-3′ and 5′-CCAAATCTCAAAGTACGCACAAACGG-3′; TRAIL-R3, 5′-GAAGAATTTGGTGCCAATGCCACTG-3′ and 5′-CTCTTGGACTTGGCTGGGAGATGTG-3′; TRAIL-R4, 5′-CTTTTCCGGCGGCGTTCATGTCCTTC-3′ and 5′-GTTTCTTCCAGGCTGCTTCCCTTTGTAG-3′; OPG, 5′-GTGACGAGTGTCTATACTGCA-3′ and 5′-ATCCTCTCTACACTCTCTGCG-3′; and $FLIP_L$, 5′-AATTCAAGGCTCAG-AAGCGA-3′ and 5′-GGCAGAAACTCTGCTGTTCC3′. The annealing temperatures were 60°C, 61°C, 61°C, 64°C, 60°C, 58°C, and 60°C, respectively.

Samples were amplified for 35 cycles using the FTS-IS thermal sequencer (Corbett Research, Mortlake, Australia). The program consisted of: 1 cycle at 96°C, 58–65°C, and 72°C for 1 min; 35 cycles of 96°C for 30 s, 58–65°C for 35 s, and 72°C for 1 min; and finally 10 min at 72°C. PCR-amplified products were run on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and were visualized under UV light.

[5] Available at http://www.genome.wi.mit.edu//cgi-bin/primer/primer3.cgi.

**DNA Sequencing.** The OPG PCR product was cloned in P-GEM T (Promega, Madison, WI) and subjected to thermocycle sequencing reactions, based on the dideoxy termination method (31). Likewise, the $FLIP_L$ PCR product was sequenced with the same primers used for amplification. The sequencing reactions were determined by using a Perkin-Elmer Catalyst 800 and an automated 377 DNA sequencer (Applied Biosystems, Foster City, CA) at the Sydney University and Prince Alfred Macromolecular Analysis Center (Sydney, New South Wales, Australia).

**Southern Blotting.** Three micrograms of genomic DNA from four different melanoma cell lines were digested with *Hin*dIII, and the resulting DNA fragments were separated by agarose gel electrophoresis, stained with ethidium bromide, and transferred to a Hybond N+ nylon membrane (Amersham, Castle Hill, NSW Australia) with a VacuGene Pump device from Pharmacia Biotech (Castle Hill, NSW Australia). While the DNA was being transferred to the membrane, the agarose gel was treated as follows: 0.25 M HCl for 15 min and 0.4 M NaOH for 2 h at 50 mbar. The membrane-bound DNA was treated with 2× SSC for 5 min at room temperature.

The TRAIL-R1 and TRAIL-R2 oligonucleotide primers described in the previous section were used to amplify both probes using a digoxigenin labeling and kit (Boehringer Mannheim, Castle Hill, NSW Australia). Membrane hybridization and detection were performed under high-stringency conditions using a digoxigenin DNA detection system. The protocol and buffers used were those described by the manufacturer with the following modification: the chemiluminescence from the labeled membrane was captured by a Kodak (Rochester, NY) digital science Image Station 440CF using a single exposure of 30 min.

**Flow Cytometry.** Analysis was carried out using a Becton and Dickinson (Mountain View, CA) FACScan flow cytometer. Appropriate concentrations of MAbs were added to the cells in 100 μl of PBS containing 20% human A serum and incubated for 7 min at room temperature. Cells were either washed twice with PBS and analyzed, if they were directly labeled, or if they were indirectly labeled, cells were then incubated with $F(ab')_2$ fragment affinity isolated FITC-conjugated sheep antimouse immunoglobulin (Silenus; Amrad Biotech, Boronia, Victoria, Australia) plus 20 μl of 100% human serum to block Fc receptors for 7 min at room temperature. A minimum of 5000 cells was analyzed. Studies on permeabilized cells were similar to the methods of

Table 1 *Sensitivity to TRAIL in relation to expression of TRAIL-R and FLIP mRNA expression in melanoma cell lines*

| Cell line | % apoptosis induced by TRAIL | TRAIL-R | | | | OPG | $FLIP_L$ |
|---|---|---|---|---|---|---|---|
| | | R1 | R2 | R3 | R4 | | |
| Mel0538 | 0 | – | – | – | – | – | – – |
| Mel JS | 0 | – | – | – | – | – | – |
| Mel SP | 0 | – | – | + | – | – | – |
| KM3 | 0 | – | – | + | – | – | – |
| Me 3.1 | 19 | – | + | – | – | – | + |
| Mel RMu | 15 | + | + | – | – | + | + |
| Mel MC | 21 | + | + | – | – | + | + |
| SKMel 28 | 20 | + | + | + | – | – | + |
| Mel RM | 76 | – | + | + | – | + | + |
| IgR3 | 21 | + | + | + | – | + | + |
| Mel MM | 4 | + | + | + | + | + | + |
| MM200 | 83 | + | + | + | + | + | + |
| Mel007 | 0 | + | + | + | + | + | – |
| Me4405 | 61 | + | + | + | + | + | + |
| Mel FH | 24 | + | + | + | + | + | + |
| Mel WB | 15 | + | + | + | + | + | + |
| Mel CV | 54 | + | + | + | + | + | + |
| Mel JG | 100 | + | + | + | + | + | + |
| Mel JC | 38 | + | + | + | + | + | + |
| Mel JF | 57 | + | + | + | + | + | + |
| Mel MS | 12 | + | + | + | + | + | + |
| Mel LT | 15 | + | + | + | + | + | + |
| Mel KN | 27 | + | + | + | + | + | + |
| Mel JB | 3 | + | + | + | + | + | + |
| Mel AT | 0 | + | + | + | + | + | + |
| Mel MI | 0 | + | + | + | + | + | + |
| Mel GL | 71 | + | + | + | + | + | + |
| A2058 | 19 | + | + | + | + | + | + |
| Melanocytes | 0 | + | + | + | + | – | + |
| Colo-205 | 50 | + | + | + | + | + | + |
| Jurkat | 78 | + | + | + | + | – | + |
| K562 | 20 | + | + | + | + | – | + |

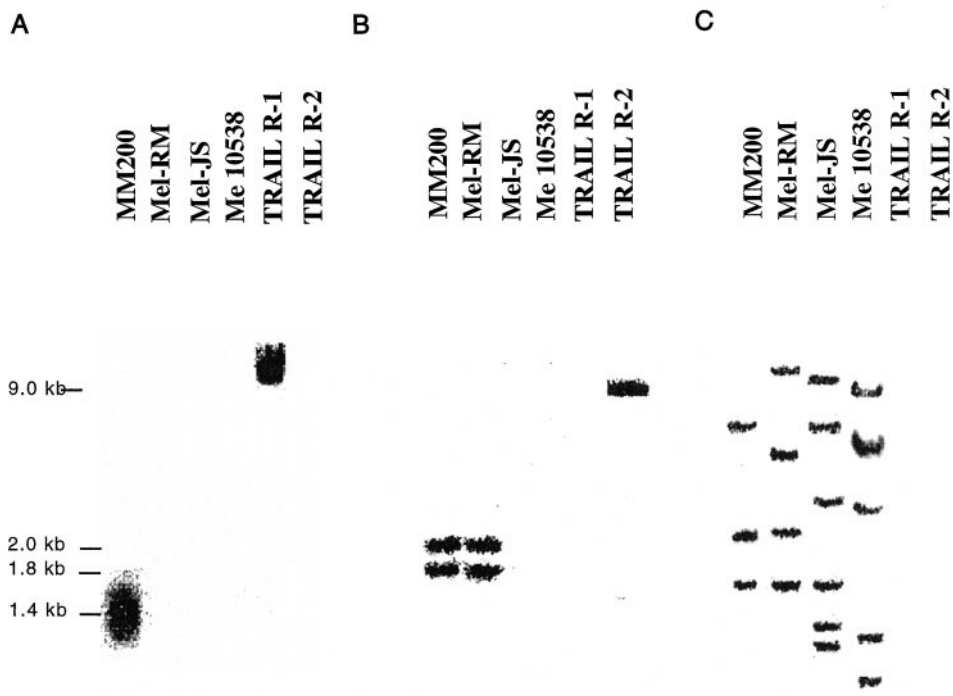


Fig. 2. Southern blots with TRAIL-R1 (*A*) and TRAIL-R2 (*B*) cDNA probes on chromosomal DNA from four melanoma cell lines. The double bands in *B* indicate that the probe is detecting both exon 9 and untranslated regions in the chromosome. TRAIL-R1 and -R2 are PCR products of the genes. Mel JS and Me10538 were negative for both genes, and Mel RM was negative for TRAIL-R1. *C*, the DNA was reacted with a probe against $\beta$-actin to indicate that equivalent amounts of DNA were loaded into each lane.

Jung *et al.* (32). The cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% saponin in permeabilization buffer, and the Ab to FLIP was added for 30 min at 4°C. The cells were washed and then stained with FITC-labeled $F(ab)_2$ fraction of affinity-isolated sheep antirabbit immunoglobulin (Silenus) at 1:100 dilution for 30 min at 4°C. After washing the cells were analyzed by flow cytometry. The TRAIL-R-negative Me10538 cell line was included as a negative control in all studies on permeabilized cells.

**Apoptosis.** Apoptotic cells were determined by the propidium iodide method (33). In brief, melanoma cells were adhered overnight in a 24-well plate (Falcon 3047; Becton Dickinson, Lane Cove, Australia) at a concentration of $1 \times 10^5$ cells/well in 10% FCS. Cells in suspension were added on the day of the assay. Medium was removed, and 500 $\mu$l of fresh medium plus 10% FCS containing the appropriate MAb were added for 30 min at 37° before the addition of TRAIL, FasL, CD40 ligand, or TNF-$\alpha$. Cells were incubated for a further 24 h at 37°C, the medium was removed, and adherent and suspended cells were washed once with PBS. The medium and PBS were placed in $12 \times 75$ mm Falcon polystyrene tube and centrifuged at $200 \times g$. One ml of a hypotonic buffer (50 $\mu$g/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma) was added directly to the cell pellet of cells grown in suspension or to adhered cells in the 24-well plate and gently pipetted off, then added to the appropriate cell pellet in the Falcon tube. The tubes were placed at 4°C in the dark overnight before flow cytometric analyses. The propidium iodide fluorescence of individual nuclei was measured in the red fluorescence using a FACScan flow cytometer (Becton Dickinson), and the data were registered in a logarithmic scale. At least $10^4$ cells of each sample were analyzed. Apoptotic nuclei appeared as a broad hypodiploid DNA peak, which was easily distinguished from the narrow hyperdiploid peak of nuclei in the melanoma cells.

**Western Blot Analysis.** The melanoma cells were harvested by Trypsinization and lysed in cell lysis buffer (34) containing phenylmethylsulfonyl fluoride, leupeptin, and other protease inhibitors for 1 h on ice. Approximately 3 million cell equivalents were loaded per track, and the protein concentration in each track was checked by visualization with Ponceau-S dye. $FLIP_L$ was detected using AL129 polyclonal Ab which was a gift from Dr. J. Tschopp raised against the 197 $NH_2$-terminal amino acids of human FLIP (fused to a flag-tag; Ref. 22). A $M_r$ of 55,500 was determined for $FLIP_L$ using ANGIS nip (30). Supernatants after centrifugation were subjected to 12.5% SDS-PAGE. Blotting and detection were performed as described by Radford *et al.* (35), with the following modifications: primary Ab was diluted to 1:200, and detection was performed using Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products, Boston, MA) and exposed on to Hyper MP autoradiography film (Amersham).

## RESULTS

**Detection of TRAIL-R, OPG, and FLIP by RT-PCR.** The PCR product sizes for TRAIL-R, OPG, and $FLIP_L$ and $FLIP_S$ are shown in Fig. 1. TRAIL-R1–R4 products had the expected sizes, as reported elsewhere (29). The OPG product had a size of 486 bp. The product was sequenced and shown to correspond to OPG between 345 and 793 bp. $FLIP_L$ had a product size of 226 bp, and sequencing of the products confirmed that they were identical to $FLIP_L$ between 1552 and 1742 bp in the database.

**TRAIL-R mRNA Expression in Melanoma Cell Lines.** A summary of the studies on 28 melanoma lines, melanocytes, Jurkat, K562, and Colo-205 lines is shown in Table 1. All results were confirmed in at least two assays. The melanoma cell lines have been arranged to illustrate the variable expression of TRAIL-R. Two of the lines, Me10538 and Mel JS, were devoid of mRNA for all TRAIL-R, whereas three expressed mRNA only for one of the receptors. Two lines, Mel RMu and Mel MC, expressed only TRAIL-R1 and -R2 and OPG receptors, and three lines expressed TRAIL-R3 with or without TRAIL-R1 and -R2. The majority (17 of 28) of the lines, however, expressed mRNA for all of the receptors, including that for OPG.

The four cell lines without death receptors (TRAIL-R1 and -R2) were resistant to TRAIL-R-induced apoptosis. The presence of mRNA for decoy receptors had no relation to sensitivity of the lines to TRAIL. There was also wide variability in sensitivity to TRAIL-induced apoptosis in the lines that expressed mRNA for all of the receptors.

We examined whether mRNA for FLIP may correlate inversely with TRAIL-induced apoptosis, but this was not apparent by PCR assays. Practically all of the lines expressed mRNA for FLIP, except those with no or only one TRAIL-R (Me10538, Mel JS, Mel SP, and KM3).

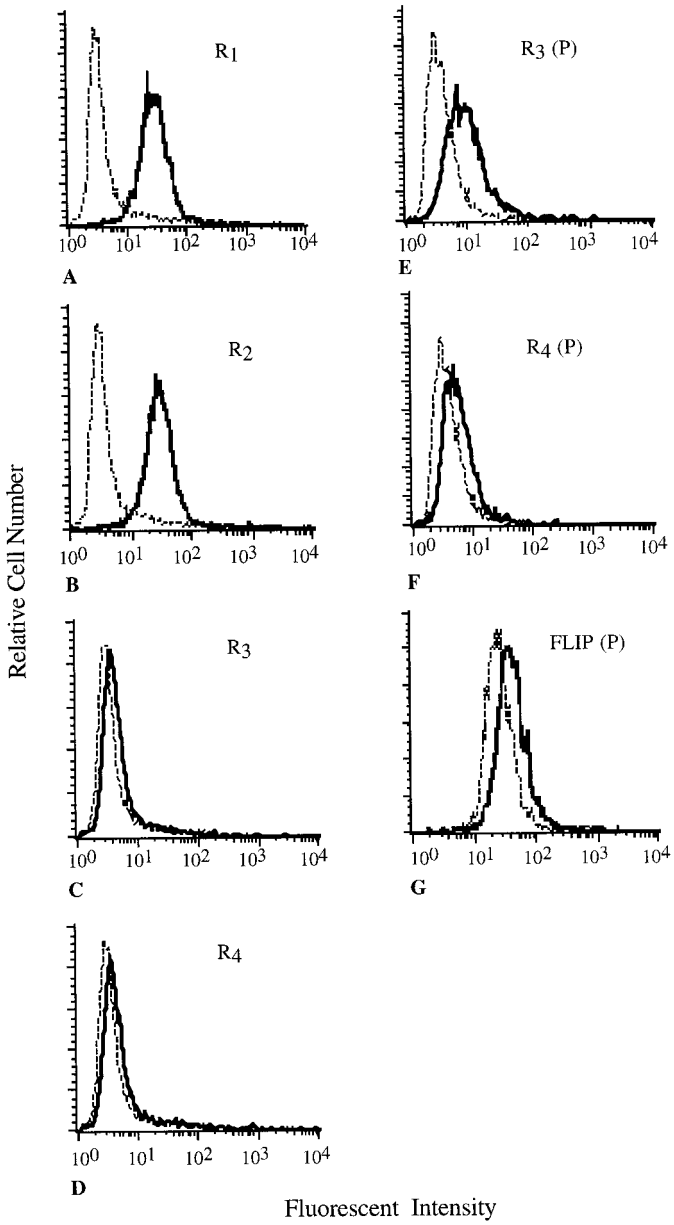


Fig. 3. Flow cytometric analysis of TRAIL-R expression on the Me4405 cell line. *A–D*, TRAIL-R1–R4 cell surface expression, respectively. *E* and *F*, TRAIL-R3 and -R4 expression, respectively, in permeabilized cells. *G*, FLIP expression within permeabilized Me4405 cells. - - - - -, isotype control histograms; ——, MAb.

**Southern Analysis of Genes for TRAIL-R1 and -R2 Receptors.** We examined whether the failure to detect TRAIL-R1 and R2 receptors may result from failure to transcribe the genes concerned or represent loss of genes from the melanoma cell lines. As shown in Fig. 2, Southern analysis of DNA from the melanoma cell lines MM200, Mel RM, Me10538, and Mel JS with probes for TRAIL-R1 and R2 revealed genes for TRAIL-R1 in the MM200 line but not in Mel RM, Mel JS, or Me10538. TRAIL-R2 was detected in MM200 and Mel RM but not in Me10538 or Mel JS. These results are consistent with those obtained by RT-PCR assays for mRNA shown in Table 1.

**Examination of TRAIL-R and FLIP Expression by Flow Cytometry.** In view of the poor correlation shown between TRAIL-R and FLIP mRNA expression with susceptibility to TRAIL-induced apoptosis, it was examined whether TRAIL-R and/or FLIP protein expression may correlate with apoptosis. Representative flow cytometer histograms for the four TRAIL-Rs and FLIP are shown in Fig. 3. A summary of representative studies on at least two occasions on 20 melanoma lines is shown in Table 2. TRAIL-R2 protein was expressed on all 20 lines that expressed mRNA for TRAIL-R2, and TRAIL-R1 protein was detected on 12 of 15 lines with mRNA for TRAIL-R1. In contrast, only 5 of 17 lines with mRNA for TRAIL-R3 and 3 of 12 with mRNA for TRAIL-R4 had detectable protein expression. Regression analyses were carried out to examine the correlation of the protein expression of TRAIL-R and FLIP with apoptosis induced by TRAIL. As shown in Fig. 4, there was a correlation between TRAIL-R1 ($r = 0.53$) and R2 expression ($r = 0.77$) and apoptosis but not with TRAIL-R3 or R4 expression. This lack of correlation is further shown by studies on individual lines, *e.g.*, cells from the Me4405 cell line were sensitive to TRAIL-induced apoptosis but had the highest level of expression of TRAIL-R3 and -R4 and FLIP. Melanocytes appeared to express only TRAIL-R2 receptors, despite having mRNA for all four receptors.

**TRAIL-R Expression within Melanoma Cells.** Melanoma lines that failed to express TRAIL-R1 on their surface, MM200, Mel LT, and Mel AT, were examined after permeabilization of their membranes. As shown in Table 2, these lines had the TRAIL-R1 proteins in the cells at comparable levels to cell lines that express TRAIL-R1 on their surface (*e.g.*, Mel FH). Permeabilized melanoma cells that had surface expression of R1 had similar or higher levels than nonpermeabilized cells, *e.g.*, Me4405, Me1007, and Mel JG. Studies on TRAIL-R3 and -R4 expression within permeabilized melanoma cells showed an even more marked disparity compared to membrane expression. As shown in Table 2, six lines that were negative for surface expression of TRAIL-R3 or -R4 had high levels of the receptors within the cells (*e.g.*, Mel FH and MM200). Cell lines with no mRNA for the receptors, *e.g.*, Me10538, acted as negative controls and no intracellular staining was detected in these cells.

**Expression of FLIP in Melanoma.** The Ab against FLIP detected a protein of $M_r$ 55,000, as expected (22) in Western blots of the Me4405, Mel FH, and MM200 cell lines but not in extracts from IgR3 and Mel CV and the Jurkat T-cell line (Fig. 5). These results were similar to the detection of FLIP by flow cytometry on permeabilized melanoma cells shown in Table 2, *e.g.*, Me4405, Mel FH, and MM200 had FLIP detectable in permeabilized cells and in Western blots, but FLIP was not detectable in Jurkat, Mel CV, and IgR3 by either method. Fig. 4 indicates that the level of FLIP expression did not show an inverse correlation with TRAIL-induced apoptosis. Permeabilized melanocytes also did not have detectable levels of FLIP (Table 2).

**TRAIL-R Expression on Clones of Melanoma Cells.** We examined whether variation in sensitivity to TRAIL between lines may reflect variability of TRAIL-R expression on clones of cells within each cell line. To examine this, clones of cells from the IgR3 cell line were established and tested for susceptibility to apoptosis and TRAIL-R expression. The results of one such experiment are shown in Table 3. There was marked variation in sensitivity to TRAIL among the clones, with percentage apoptosis ranging from 13.6 (clone F2) to 100% (clone C3). There was no difference in TRAIL-R and FLIP expression by PCR, but TRAIL-R1 and R2 protein expression showed significant variation between the clones. These variations did not correlate with sensitivity to apoptosis, and the clone with the lowest sensitivity to TRAIL (F2) had the highest TRAIL-R expression. These results were repeated on two occasions with the same results, *i.e.*, the clones appeared to

Table 2 *Expression of TRAIL-R and FLIP in melanoma cell lines*

| Cell line | % apoptosis induced by TRAIL | TRAIL-R | | | | FLIP[a] |
|---|---|---|---|---|---|---|
| | | R1 | R2 | R3 | R4 | |
| Mel0538 | 0 | 0 (0)[b] | 0 (0) | 0 (0) | 0 (0) | 0 |
| Mel JS | 0 | 0 | 0 | 0 | 0 | 0 |
| Mel SP | 0 | 0 | 0 | 0 | 0 | 0 |
| KM3 | 0 | 0 | 0 | 18.6 | 0 | 0 |
| Mel RMu | 15 | 72.9 | 50.5 | 0 | 0 | 0 |
| SKMel 28 | 20 | 18.6 (34) | 32.2 (49) | 0 (30) | 0 (0) | 0 |
| Mel RM | 76 | 0 | 73.2 | 0 | 0 | 10.5 |
| IgR3 | 21 | 79.2 (73) | 79.9 (71) | 0 (68) | 0 (0) | 0 |
| MM200 | 83 | 0 (19) | 76.1 (69) | 5.2 (60) | 0 (39) | 5.9 |
| Mel007 | 0 | 13.5 (31) | 10.4 (44) | 3.2 (45) | 0 (33) | 0 |
| Me4405 | 61 | 91 (87) | 89.8 (86) | 17.5 (72) | 5.3 (11) | 34.8 |
| Mel FH | 24 | 10.7 (11) | 67.2 (68) | 0 (40) | 0 (29) | 13.2 |
| Mel CV | 54 | 32.8 | 51.1 | 0 | 0 | 0 |
| Mel JG | 100 | 65.8 (75) | 87.4 (81) | 0 (51) | 0 (16) | 0 |
| Mel LT | 15 | 0 (9) | 63.4 (58) | 0 (35) | 0 (20) | 30.7 |
| Mel AT | 0 | 0 (6) | 40.2 (38) | 0 (36) | 0 (26) | 18.8 |
| Mel GL | 71 | 30 | 70.5 | 0 | 0 | 16.1 |
| Melanocytes | 0 | 0 | 50 (50) | 0 (0) | 0 (0) | 0 |
| Colo 205 | 19 | 44.3 | 40.5 | 2.0 | 2.5 | 0 |
| Jurkat | 78 | 70.20 | 50.1 | 0 | 0 | 0 |
| K562 | 20 | 26.5 | 38.4 | 2.9 | 6.0 | 12 |

[a] Results obtained on permeabilized cells.
[b] Numbers in parentheses indicate results on permeabilized cells.

express stable levels of receptor expression and sensitivity to TRAIL-induced apoptosis.

## DISCUSSION

Previous studies have suggested that sensitivity to TRAIL-induced apoptosis is regulated by the presence or absence of two receptors

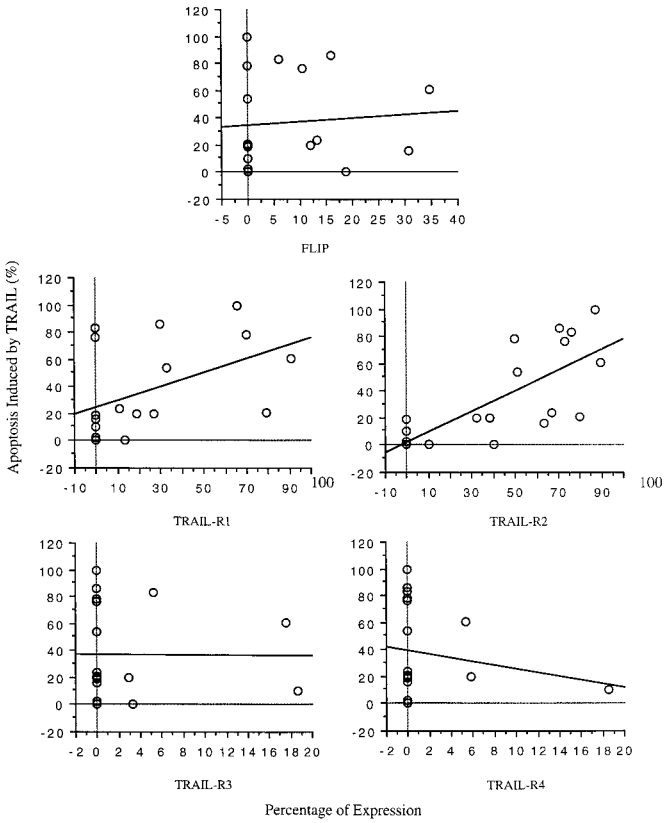


Fig. 4. Regression analysis of the relation between TRAIL-induced apoptosis of melanoma cell lines and TRAIL-R cell surface expression or FLIP expression in permeabilized cells. Regression coefficient for relation between apoptosis and TRAIL-R1 and -R2 expression were 0.53 and 0.77, respectively ($P = 0.0008$ and 0.05, respectively.)

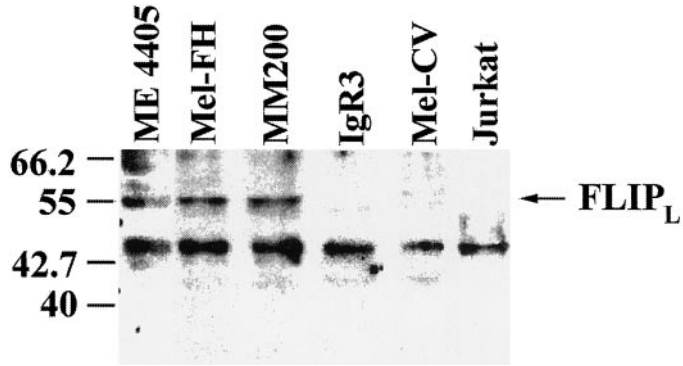


Fig. 5. Western blot showing detection of FLIP at $M_r$ 55,000 by the polyclonal Ab AL129 in extracts of the melanoma lines Me4405, MM200, and Mel FH but not in IgR3, Mel CV, or Jurkat cell lines. Isotype control Ab was unreactive. The $M_r$ 49,000 band is nonspecifically detected by the secondary Ab.

(TRAIL-R1 and -R2) with death-inducing ability and two receptors with decoy activity (TRAIL-R3 and -R4) that inhibit the death signals (3, 15). We have shown previously that TRAIL but not other members of the TNF family can induce apoptosis in a wide range of melanoma cells (26). Similar findings were reported by Griffith *et al.* (29), who also found that sensitivity of melanoma cells to TRAIL did not correlate with their expression of mRNA for the decoy receptors in that some of the sensitive lines expressed mRNA for TRAIL-R3 and/or -R4.

These studies on a larger panel of melanoma lines extend these findings in several areas.

(*a*) They show that there is considerable variability in TRAIL-R expression on melanoma lines, which results from at least two factors. One is the loss of genes coding for the receptors. This was apparent for both TRAIL-R1 and -R2 in 4 of the 28 (14%) melanoma lines by RT-PCR and confirmed in two of the lines by Southern analysis. Two of the lines appeared to have lost genes for all of the TRAIL-Rs, and in three lines, all but one of the receptors had been lost. All four TRAIL-Rs were reported to be clustered on 8p22–21 (12), and it is, therefore, possible that this segment or part thereof was lost from the cell lines and selected in the patients by the survival pressure exerted by TRAIL in their environment.

The second factor underlying variability of TRAIL-R expression

Table 3 *Sensitivity to TRAIL-induced apoptosis in relation to TRAIL-R and FLIP expression in clones of melanoma cells from the IgR3 melanoma line*

| IgR3 clones | % apoptosis induced by TRAIL | | TRAIL-R and FLIP mRNA | | | | | | TRAIL-R and FLIP by flow cytometry | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100 ng | 500 ng | R1 | R2 | R3 | R4 | OPG | FLIP | R1 | R2 | R3 | R4 | FLIP |
| IgR3 | 21 | | + | + | + | − | + | + | 79.2 | 79.9 | 0 | 0 | 0 |
| B6 | 31 | 39 | + | + | + | − | − | + | | | | | |
| B9 | 98 | 100 | + | + | + | − | + | + | 74 | 50.12 | 0 | 0 | 0 |
| C3 | 100 | 100 | + | + | + | − | + | + | 58 | 61.71 | 0 | 0 | 0 |
| C5 | 47 | 66 | + | + | + | − | + | + | | | | | |
| C6 | 76 | 83 | + | + | + | − | + | + | | | | | |
| C11 | 53 | 100 | | | | | | | | | | | |
| E2 | 42 | 72 | | | | | | | | | | | |
| E5 | 25 | 38 | + | + | + | − | + | + | 57.52 | 47.82 | 0 | 0 | 0 |
| F2 | 14 | 21 | + | + | + | − | + | + | 78.69 | 80.68 | 0 | 0 | 0 |

appeared to occur at the protein level. Whereas all of the melanoma lines with mRNA for TRAIL-R2 had TRAIL-R2 on their surface, 3 of 15 lines with mRNA for TRAIL-R1 did not express the receptor on the cell surface. This phenomenon was even more marked for TRAIL-R3 and -R4, in that only 5 of 17 and 3 of 12 cell lines, respectively, that had mRNA for the receptors actually expressed the receptor on their surface. It was of much interest to find that many of the cell lines that were negative for receptors on their surface nevertheless appeared to have significant levels within their cytoplasm. Confocal microscopy indicates the TRAIL-R2 receptors are clustered in organelles, similar to the Golgi apparatus, whereas TRAIL-R3 and -R4 are located in the nucleus (data not shown). Previous studies have shown that Fas, the receptor for FasL, is located predominantly within the cell in certain cell types and is rapidly relocated to the cell surface on activation of p53 (36). To our knowledge, this is the first to suggest that TRAIL-R expression may be regulated in a similar manner at a posttranslational level. Further study on this aspect as well as on additional regulatory mechanisms that may operate at a transcriptional level to regulate TRAIL-R expression is needed.

(*b*) These studies show that the level of TRAIL-R1 and, particularly, TRAIL-R2 protein expression on the melanoma lines correlated well with their sensitivity to TRAIL-induced apoptosis. However, although this applied to the cell lines overall, certain cell lines were resistant to TRAIL, despite moderate levels of TRAIL-R1 or -R2 expression. This was also evident in clones of melanoma cells established from a cell line. These exhibited different levels of TRAIL-R1 and -R2 protein expression that did not correlate with the marked variation in sensitivity of the clones to TRAIL-induced apoptosis. These findings suggest that the level of TRAIL receptor expression alone is not sufficient to account for sensitivity to TRAIL induced apoptosis and that additional factors that regulate intracellular pathways leading to apoptosis are involved. A prime candidate for this role is the protein FLIP, which binds to caspase 8 and prevents activation of the downstream events leading to apoptosis (22). FLIP was shown to regulate susceptibility of activated T cells to FasL-induced apoptosis and to block TRAIL-induced apoptosis. Subsequent studies on melanoma cell lines indicated that FLIP protein levels in Western blots correlated with susceptibility to TRAIL-induced apoptosis (29).

In these studies mRNA for the long form ($M_r$ 55,000) of FLIP was detected in all but 5 of the 28 melanoma lines. However, although FLIP protein expression was detected in 7 of 12 (58%) melanoma cell lines that had mRNA for FLIP, the presence and levels of protein expression did not correlate with resistance to TRAIL-induced apoptosis. Studies on tissue sections showed that 43% of primary melanoma and 63% of metastases expressed FLIP, which is comparable to results on the cell lines (unpublished data). These results may, therefore, indicate that other regulatory proteins that inhibit apoptosis, such as the NF-$\kappa$B induced IAP proteins (23), may be involved. The latter were reported to be key proteins responsible for inhibition of apoptosis following interaction with TNF-$\alpha$ or other agents that activate NF-$\kappa$B.

(*c*) To our knowledge the present studies are the first to show that a fifth receptor for TRAIL, OPG, is also expressed at the mRNA level on melanoma lines. Melanocytes did not express mRNA for OPG, but 22 of 28 melanoma lines did. OPG was shown to bind with relatively low affinity to TRAIL and to inhibit TRAIL-induced apoptosis of Jurkat cells (21). We could not demonstrate a correlation between OPG mRNA expression and susceptibility of melanoma cells to TRAIL-induced apoptosis. Further information about protein expression is needed to examine this aspect further. OPG is a secreted product and may conceivably neutralize TRAIL in the circulation of patients. The natural ligand for OPG is osteoclast differentiation factor (TRANCE/RANKL; Ref. 37), so that it might inhibit osteoclast formation around metastases. Further study of these aspects is needed.

These results appear to have important implications for subsequent clinical use of TRAIL. (*a*) The relatively high proportion of TRAIL-R negative melanoma lines suggest it will be important to assess the TRAIL-R status of tumors in the patient before therapy. (*b*) The detection of TRAIL-R melanoma with mRNA for TRAIL-R and TRAIL-R protein within the cell but not on the cell surface indicates more information is needed about regulation of TRAIL-R expression. Similarly, the presence of melanoma lines with surface expression of TRAIL that are resistant to TRAIL-induced apoptosis requires more understanding of the regulatory pathways and inhibitors controlling apoptosis induced by TRAIL.

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# Relation of TNF-related Apoptosis-inducing Ligand (TRAIL) Receptor and FLICE-inhibitory Protein Expression to TRAIL-induced Apoptosis of Melanoma

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